

**SANDWICH-CULTURED RAT HEPATOCYTES: A NOVEL *IN VITRO* MODEL TO
STUDY HEPATOBILIARY DISPOSITION OF SUBSTRATES**


by
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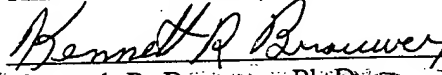
A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in
partial fulfillment of the requirements for the degree of Doctoral of Philosophy in the School
of Pharmacy.

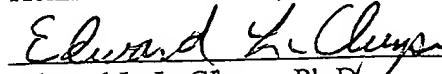
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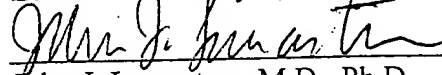
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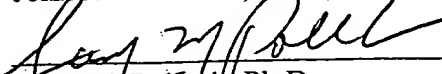
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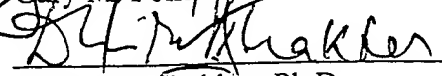
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ABSTRACT

Xingrong Liu: Sandwich-Cultured Rat Hepatocytes: A Novel *In Vitro* Model to Study

Hepatobiliary Disposition of Substrates

(Under the direction of Kim L. R. Brouwer, Pharm.D., Ph.D.)

N.B. This dissertation is in the form of four free-standing manuscripts.

The objective of this dissertation research was to examine the membrane transport properties of long-term sandwich-cultured rat hepatocytes, to assess the utility of sandwich-cultured hepatocytes as an *in vitro* model to study hepatobiliary disposition, and to predict *in vivo* biliary excretion. The specific aims of this project were to (1) examine the expression and functional activity of transport proteins responsible for hepatic uptake and biliary excretion of substrates in long-term sandwich-cultured hepatocytes; (2) establish an experimental approach to quantitate the extent of biliary excretion in the sandwich-cultured hepatocytes; and (3) assess the hepatobiliary disposition of substrates and the correlation between biliary excretion in the sandwich-cultured hepatocytes and *in vivo* biliary excretion. Expression of Na⁺/taurocholate cotransporting polypeptide (Ntcp) and canalicular multispecific organic anion transporter (cMOAT) was detected in 96-hr sandwich-cultured hepatocytes by immunoblot analysis. The activity of Ntcp, the canalicular bile acid transporter, and cMOAT measured by the uptake of taurocholate, the excretion of taurocholate, and the excretion of carboxydichlorofluorescein, respectively, was maintained in 96-hr sandwich-cultured hepatocytes. The integrity of the canalicular network in sandwich-cultured hepatocytes was

maintained by tight junctions as demonstrated by confocal fluorescence and electron microscopy. Ca^{2+} depletion increased tight junction permeability and enabled substrate translocation between the canalicular and the extracellular spaces based on favorable concentration gradients. Cumulative uptake of taurocholate in 96-hr sandwich-cultured hepatocytes pre-incubated in standard buffer (intact canaliculi) or Ca^{2+} -free buffer (disrupted canaliculi) was described best by a kinetic model composed of cytosolic and bile compartments in standard buffer, but only a cytosolic compartment in Ca^{2+} -free buffer. Michaelis-Menten functions best described the uptake and biliary excretion processes. The extent of biliary excretion in the hepatocyte monolayers was quantitated by the differential cumulative uptake of substrate in the presence and absence of extracellular Ca^{2+} . Biliary clearance of model substrates measured in 96-hr sandwich-cultured hepatocytes correlated with *in vivo* intrinsic biliary clearance in rats. This dissertation research demonstrates that long-term sandwich-cultured hepatocytes maintain the expression and functional activity of transport proteins responsible for hepatic uptake and biliary excretion of substrates. Sandwich-cultured hepatocytes can be utilized in mechanistic studies of hepatobiliary disposition and can predict *in vivo* biliary excretion.

ACKNOWLEDGMENTS

I would like to express my sincere thanks to those who have helped me during my graduate education.

To Dr. Kim Brouwer, my dissertation advisor, for her support, guidance, and encouragement.

To my dissertation committee members, Drs. Edward LeCluyse, Kenneth Brouwer, John Lemasters, Gary Pollack, and Dhiren Thakker, for their precious time and insightful comments.

To Dr. Lawrence Gan, Mr. Jack Chism, Dr. Ruth Lightfoot, Ms. Jacqueline Lee, Dr. Ann LeFurgey, Dr. Ting Qing, Dr. Stephen Letrent, Ms. Pamela Amos, and Dr. Catherine Booth for their technical assistance.

To my fellow graduate students in the Lab 21 for their encouragement and friendship.

To my wife, Dr. Cuiping Chen, and my son, Anthony Liu, my parents, sister and brothers for their love, understanding, and encouragement.

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LIST OF ABBREVIATIONS

cMOAT	canalicular multispecific organic anion transporter
HBSS	Hanks' balanced salt solution
Ntcp	Na ⁺ /taurocholate cotransporting polypeptide
Oatp 1	organic anion transport polypeptide
Oct 1	organic cation transporter

CHAPTER 1

INTRODUCTION

The liver is one of the major organs involved in the detoxification and elimination of xenobiotics. Accurate evaluation of the hepatic disposition of xenobiotics is necessary to predict the pharmacologic and toxicologic effects of drugs, variations in drug disposition during disease, and drug-drug interactions. Hepatobiliary transport represents a fundamental process in the hepatic disposition of xenobiotics *in vivo* and can be the rate-limiting step in overall hepatic elimination (Klaassen and Walkins, 1984; Groothuis and Meijer, 1996). Many endogenous and exogenous compounds undergo hepatic uptake and biliary excretion via carrier-mediated transport (Oude Elferink *et al.*, 1995). In particular, primary active transport mechanisms have been shown to be responsible for the biliary excretion of anticancer drugs, endogenous bile acids, and organic anions including glutathione and glucuronide conjugates. A variety of *in vivo* and *in vitro* approaches have been utilized to study biliary excretion (Oude Elferink *et al.*, 1995). However, the existing methods may not always be used to investigate human biliary excretion, and cannot determine efficiently biliary excretion for a large number of compounds in the drug discovery and development process. Long-term primary rat hepatocytes cultured between two layers of collagen gel (sandwich configuration) maintain liver-specific function and form bile canalicular networks (Dunn *et al.*, 1991; LeCluyse *et al.*, 1994). Sandwich-cultured hepatocyte monolayers represent a potential *in vitro* model to study biliary excretion. This dissertation research was conducted to evaluate membrane transport activity in long-term sandwich-cultured hepatocytes, to examine the utility of this *in vitro* model to study hepatobiliary disposition, and to assay the ability of this model to predict *in vivo* biliary excretion.

Importance of Biliary Excretion

Assessment of *in vivo* biliary excretion is important to achieve therapeutic end-points, to anticipate variations in drug disposition during disease states, and to predict drug-drug interactions. For a drug that is excreted extensively in bile, sojourn in the body may be increased significantly by inhibition of biliary excretion with other medications. Disease states may modulate hepatobiliary transport characteristics for a drug, further altering disposition. Therefore, in order to achieve a therapeutic goal, the dose of the drug may need to be adjusted on the basis of patient-specific parameters. A drug may be excreted into bile as glucuronide conjugates. The glucuronide conjugates may be hydrolyzed by intestinal microflora to form precursors, either the parent compound or phase I metabolites, which may be re-absorbed and enter the systemic circulation. Any modulation of this process, termed enterohepatic recirculation, may alter drug plasma concentrations. For example, antibiotics can inhibit the metabolic activity of intestinal microflora, potentially inhibiting enterohepatic recirculation and decreasing plasma concentrations and pharmacologic effects (Parker *et al.*, 1980). This drug-drug interaction has been suspected to cause the failure of the oral contraceptive drug ethinylestradiol (Shenfield, 1993). An understanding of hepatobiliary drug transport can be used to multimize therapeutic effects or minimize toxicologic consequences, and to predict drug-drug interactions *in vivo*. However, the biliary transport of many drugs is virtually unknown because of the difficulty in studying this process *in vivo*, especially in humans.

Elucidation of biliary excretion properties of drug candidates also is a critical issue for drug discovery and development. Drug candidates that are excreted extensively into bile

may never achieve adequate concentrations *in vivo*. For example, many metabolically stable oligopeptides exhibit short residence times in the systemic circulation (Greenfield *et al.*, 1989; Adedoyin *et al.*, 1993; Chen and Pollack, 1997) and low bioavailability after oral administration (Ziegler *et al.*, 1985, 1991) due to rapid and extensive biliary excretion. If the biliary excretion properties of a drug candidate can be assessed in the early stages of drug discovery, the efficiency of drug development could be improved substantially. Therefore, knowledge of the biliary excretion characteristics of drug candidates in the early stages of drug development may be as important as characterization of absorption and metabolic properties in selecting drug candidates. In addition, if a data base of the extent of biliary excretion can be generated for a series of compounds, it may be possible to assess the relationship between chemical structure and biliary excretion properties. Even though biliary excretion is an important consideration in the drug discovery and development process, information regarding biliary excretion characteristics is limited for most drugs due to the complexity of hepatobiliary disposition and difficulty in studying biliary excretion processes, especially in humans.

Hepatic Membrane Transport Systems

Hepatic processing of endogenous compounds and xenobiotics involves many complex biochemical processes that presently are not well understood. Compounds in blood access hepatocyte cytosol by crossing the sinusoidal membrane via simple or facilitated diffusion or active transport. Once localized in the cytosol, compounds may efflux back to blood, bind to intracellular proteins, accumulate in organelles, be subject to hepatic

metabolism, or be excreted into bile. Therefore, hepatobiliary transport of drugs can be envisioned as a number of sequential translocation processes: sinusoidal uptake, intracellular protein binding, sequestration, metabolism, and sinusoidal egress or biliary excretion.

Hepatic Uptake

A number of transport systems localized on the basolateral domain of hepatocytes remove effectively a wide variety of compounds from sinusoidal blood. Polar compounds enter hepatocytes by membrane carrier transport or receptor-mediated endocytosis (Meijer *et al.*, 1983; Meijer, 1987). Three active transport systems that are important in the uptake of endogenous and exogenous compounds on the sinusoidal membrane have been characterized in rats. These transporters are Na⁺/taurocholate cotransporting polypeptide (Ntcp; Hagenbuch *et al.*, 1990; 1991), polyspecific organic anion transport protein (oatp 1; Jacquemin *et al.*, 1994), and organic cation transporter (oct 1, Grundemann *et al.*, 1994). Evidence suggests that these transport systems often have broad and overlapping substrate specificity.

Bile acid uptake is the most extensively studied process due to its physiological and pathological importance in bile formation. Taurocholate is a conjugated trihydroxy bile salt abundant in human and rat bile and metabolically stable (Inoue *et al.*, 1984). Taurocholate has been employed as a classic substrate to study hepatic uptake and biliary excretion. There is ample evidence that taurocholate is taken up by hepatocytes via a Na⁺-dependent mechanism (Meier *et al.*, 1988; Zimmerli *et al.*, 1989). A Na⁺-dependent taurocholate uptake system from rat liver has been expressed in *Xenopus laevis* oocytes (Hagenbuch *et al.*, 1990)

and a full-length cDNA has been cloned subsequently with this expression system (Hagenbuch *et al.*, 1991). This cDNA encodes for a protein (Ntcp) that has an unglycosylated molecular mass of 33-35 kDa. High stringency blotting of several rat tissues revealed expression only in liver tissue. Using antibodies against the gene product, Stieger *et al.* (1994) demonstrated that the molecular mass of the gene product in rat liver is 51 kDa. After deglycosylation, the apparent molecular mass of Ntcp decreased to 33.5 kDa, suggesting extensive glycosylation of the native protein. Furthermore, it has been demonstrated that the protein is localized specifically in the sinusoidal membrane. Taurocholate transport into oocytes that had been injected with Ntcp cDNA was found to be strictly Na^+ -dependent. Ntcp preferentially mediates Na^+ -dependent transport of conjugated bile salts such as taurocholate; this transport comprises the predominant fraction in hepatic bile salt uptake (Meier, 1995; Hagenbuch *et al.*, 1996). Ntcp is strongly dependent on the differentiation state of hepatocytes: Ntcp mRNA is almost undetectable in primary cultured rat hepatocytes at 72 hours after plating (Liang *et al.*, 1993). Ntcp is not expressed in human hepatoma cells (Kullak-Ublick *et al.*, 1996). With similar cloning strategy, a cDNA encoding an organic anion transport polypeptide (oatp 1) was isolated from a rat liver library by Jacquemin *et al.* (1991; 1994). Upon injection in *Xenopus laevis* oocytes, mRNA derived from this clone gave rise to Na^+ -independent taurocholate uptake, suggesting that the Na^+ -independent taurocholate carrier probably is identical to oatp 1 (Buscher *et al.*, 1987). This cDNA encoded a 71 kDa glycosylated polypeptide in the presence of canine pancreatic microsomes. In addition to mediating Na^+ -independent transport of organic anions such as bile salts and bromosulfophthalein, oatp 1 also accepts uncharged compounds, permanently

charged bulky organic cations such as N-(4',4'-azo-n-pentyl)-21-deoxyanmalinium (Meijer *et al.*, 1990), and steroids (Bossuyt *et al.*, 1996). Therefore, rat oatp 1 is a polyspecific transport system that transports a large variety of structurally unrelated and differently charged amphipathic compounds (Muller and Jansen, 1997). Grundemann *et al.* (1994) have cloned an organic cation transporter (oct 1) from a rat kidney cDNA library that is expressed in renal proximal tubule cells, hepatocytes, and small intestinal enterocytes. Oct 1 is composed of 556 amino acids and most likely functions as a basolateral transport protein for small organic cations such as tetraethylammonium, N-methyl-4-phenylphridine, and choline. There is no significantly homology between oatp 1 and oct 1 (Muller and Jansen, 1997).

Carrier-mediated transport is the major mechanism for uptake of organic anions and cations. However, it should be emphasized that passive uptake can occur for lipophilic compounds, including neutral compounds as well as both anions and cations. For instance, carboxydichlorofluorescein diacetate, a lipid soluble, anionic carboxylate readily translocates across the hepatic sinusoidal membrane by passive diffusion (Haugland, 1992).

Intracellular Disposition

The disposition of substrates in hepatocytes is influenced by (1) intracellular protein binding, (2) sequestration in organelles, and (3) biotransformation. The intracellular binding of substrates is important for net hepatic uptake and prevention of intracellular toxicity (Meijer, 1989). For organic anions, three classes of cytosolic binding proteins have been identified (Ketterer *et al.*, 1967; Levi *et al.*, 1969; Morey and Litwack, 1969; and Stolz *et al.*, 1987). Glutathione S-transferase B, (ligandin, Y protein) is the major binding protein for

organic anions such as bilirubin and cortisol metabolites, and constitutes 4-5% of the cytosolic protein (Levi *et al.*, 1969). Hepatic fatty acid-binding protein (Z protein) is another major binding protein for a variety of organic anions, including bilirubin, steroid sulfates, and very hydrophobic uncharged compounds such as sex steroids. Z protein constitutes ~2% of cytosolic protein (Ketterer *et al.*, 1976). For bile acids, the major cytosolic binding protein is 3 alpha-hydroxysteroid dehydrogenases (Y' protein; Stolz *et al.*, 1987).

Intracellular sequestration in organelles represents a critical process in determining the sojourn and distribution of substrates in cytosol, especially for cationic compounds. The organelles that are able to accumulate cations are endosomes/lysosomes, mitochondria, and nucleus due to acidic conditions, negative membrane potential, and/or the existence of negatively charged DNA molecules (Meijer, 1989). Studies suggest that mitochondria constitute a major component of the hepatic storage compartment for organic cations (Steen *et al.*, 1993).

Drug biotransformation or metabolism, an essential process of drug disposition in liver, is generally seen as a detoxification process, resulting in less active and less toxic compounds. However, increasing evidence suggests that drug metabolism can form highly toxic products (DeBaun *et al.*, 1970; Weisburger and Weisburger, 1973; Mulder *et al.*, 1986). Although non-parenchymal endothelial and Kupffer cells contain drug-oxidation enzymes, drug metabolism of small molecules in the liver predominantly occurs in the hepatocytes (Marker *et al.*, 1986). The metabolism of drugs can be divided into phase I and phase II biotransformation reactions by which drugs are "prepared" for excretion. Phase I metabolism, predominantly catalyzed by cytochrome P450 mixed-function oxidase system,

includes oxidation, reduction, and hydrolytic reactions, which often produce more reactive species of the drugs for further conjugation reactions. Phase II metabolism, catalyzed by uridine diphosphate glucuronosyltransferases, sulfotransferases, and glutathione S-transferases, consists of conjugation with glucuronic acid, sulfuric acid, or glutathione, respectively. Phase II metabolism results in formation of hydrophilic metabolites. Most of the phase I and some of the phase II enzymes are embedded in the lipid environment of the smooth endoplasmic reticulum. Almost all of the enzymes involved in biotransformation have multiple isoforms. The expression of many individual isoforms can be induced by xenobiotics (Parkinson, 1996; Ortiz de Montellano, 1995; Kato *et al.*, 1989)

Biliary Excretion

For many cholephilic compounds, excretion across the canalicular membrane is the rate-limiting step in overall biliary excretion and represents the most important concentrative step. At least four primary active transport systems on the canalicular membrane have been reported. These transporters belong to the ATP binding cassette (ABC) superfamily of transporter proteins. The canalicular bile acid transporter has been postulated based on transport studies in isolated membrane vesicles (Adachi *et al.*, 1991), but the gene has not been cloned nor has the transport protein been characterized. However, recently a new ABC cassette protein on the canalicular membrane, Sister of P-glycoprotein (Childs *et al.*, 1996; Muller *et al.*, 1996), was discovered, but demonstration of its substrate specificity has been elusive. Gerloff *et al.* (1998) reported that Sister of P-glycoprotein may be the ATP-dependent bile acid carrier on the canalicular membrane. Another important transport system

is the canalicular multispecific organic anion transporter (cMOAT), which preferentially transports organic anions other than bile acids (Ishikawa *et al.*, 1990; Akerboom *et al.*, 1991). Recently, the cDNA for rat cMOAT (the product of *mrp2* gene) was isolated, and it has been shown that cMOAT was expressed in the canalicular membrane of normal but not TR⁻ rats (Paulusma *et al.*, 1996). TR⁻ rats are a mutant strain of Wistar rats that are unable to excrete cMOAT substrates into bile. Rat cMOAT contains 1541 amino acids with a calculated molecular mass of 173,318 Da and 11 predicted N-glycosylation sites (Buchler *et al.*, 1996). The apparent molecular mass of cMOAT in rat liver is approximately 190 kDa due to glycosylation (Buchler *et al.*, 1996, Trauner *et al.*, 1997). Trauner *et al.* (1997) noted that the molecular mass of cMOAT increased 10-15 kDa in common bile duct ligated rats and suggested that post-translational processing of this protein may be altered during cholestasis. Two other important transporters on the canalicular membrane are the multiple drug resistance (MDR) gene products, *mdr1a/1b* and *mdr2*. The *mdr1a/1b* gene product (P-glycoprotein) predominantly mediates export of hydrophobic cations (Spoelstra *et al.*, 1994); and the *mdr2* gene product acts as a “flippase”, translocating phospholipids through the canalicular membrane (Smit *et al.*, 1993).

Techniques to Study Hepatobiliary Transport

A variety of *in vivo* and *in vitro* liver preparations have been utilized to study hepatobiliary transport. Each method has advantages as well as limitations. Combinations of methods often are required to elucidate the mechanisms of interest for specific transport processes. Alternatively, certain methods are more suitable to investigate specific aspects of

hepatobiliary transport. The majority of hepatic transport studies, *in vivo* and *in vitro*, have been performed in laboratory rats. Six common methods of studying hepatobiliary transport include the rat *in vivo*, isolated perfused livers, isolated hepatocytes, short-term cultured hepatocyte couplets, membrane vesicles, and cloned or reconstituted carrier proteins.

In intact animals, hepatobiliary clearance can be evaluated under normal physiological conditions. The recent availability of mutant animals with selective deficiencies in transport function has been very useful in identification of transporter substrate specificity. A good example is the demonstration of carboxydichlorofluorescein as a cMOAT substrate with TR⁻ rats (Kitamura *et al.*, 1990). However, blood protein binding, the existence of other elimination organs, and tissue distribution makes the intact animal a complex model for examining mechanisms of hepatobiliary transport of xenobiotics. Studying drug transport in the isolated perfused liver has the advantages of the intact liver *in vivo* but eliminates extrahepatic influences; however, this model lacks experimental efficiency. Normally the viability of isolated perfused rat livers declines rapidly after 2 hours; thus, this preparation cannot be used for long-term biliary excretion studies. In addition, this *in vitro* approach cannot be used to study human biliary excretion for obvious ethical reasons.

Plasma membrane vesicles are a useful tool to investigate transport function on the sinusoidal and canalicular domains. However, potential problems with this model system include the orientation of the vesicles and cross-contamination with other plasma membranes or endosomal membranes. In order to assess the molecular features of membrane transport, many attempts have been made to isolate and characterize the carrier proteins involved. The

most successful results have been obtained with expression cloning in *Xenopus laevis* oocytes for studying carriers involved in uptake of drugs (Hagenbuch *et al.*, 1990; 1991). However, investigation of excretory transport systems is hampered by the existence of relatively high endogenous excretion activity in *Xenopus laevis* oocytes (Shneider *et al.*, 1993).

Freshly isolated hepatocyte suspensions have been widely used to investigate mechanisms of drug transport. Isolated hepatocytes exhibit transport characteristics similar to those of isolated perfused livers and livers *in vivo* (Oude Elferink *et al.*, 1995). This model system is very versatile for estimation of the Michaelis-Menten constant (K_m) and maximal uptake capacity (V_{max}), and determination of the driving forces for uptake, as well as competition by other drugs and the influence of protein binding (Berry *et al.*, 1992). The loss of cell polarity, redistribution of canalicular membrane proteins over the entire cell surface (Groothuis *et al.*, 1981), and difficulty in discerning excretion via the sinusoidal versus the canalicular membrane makes isolated hepatocytes less suitable for the study of drug biliary excretion, although biliary excretion processes may be detected in this system (Oude Elferink *et al.*, 1990; Studenberg and Brouwer, 1993).

Short-term cultured hepatocyte couplets maintain a canalicular space, representing a useful *in vitro* model to study primary bile excretion processes. Canalicular bile secretion has been studied by applying fluorescence (Verkade *et al.*, 1992) and confocal fluorescence microscopy (Graf and Boyer, 1990) in hepatocyte couplets. This model system requires fresh hepatocyte preparations and the use of fluorescent compounds, thus limiting the applications of this technique.

Long-Term Sandwich-Cultured Hepatocytes: a Potential *In Vitro* Model to Study Hepatobiliary Disposition and Biliary Excretion

Conventional long-term cultured hepatocytes and hepatocyte-derived cell lines are not adequate models to study drug transport. Under conventional culture conditions, hepatocytes rapidly dedifferentiate and exhibit decreased, or even a lack of, expression of membrane transporters and other liver-specific functions, while hepatocyte-derived cell lines often lack liver-specific transport function (Petzinger and Frimmer, 1988; Kukongviriyapan and Stacey, 1989). For instance, Na⁺-dependent taurocholate uptake deteriorated in conventionally cultured hepatocytes within 3 days to 4% of the level of hepatocytes cultured for 3 hours (Liang *et al.*, 1993). Many attempts to improve culture conditions have been made in order to maintain liver-specific function and restore hepatocyte morphology. In the presence of high concentrations of nicotinamide, a network of canaliculi formed in cultures of primary adult rat hepatocytes on collagen gels (Gebhardt and Jung, 1982). Functional bile canaliculi also were observed in cultures of the hybrid cell line WIF12-1; however, only a fraction of the cells formed bile canalicular structures (Cassio *et al.*, 1991). For the most part, under conventional culture conditions, *in vitro* hepatic cell models generally do not form the extensive and continuous canalicular system observed *in vivo*. By mimicking the native extracellular matrix geometry, Dunn *et al.* (1991) showed that hepatocyte cultures maintained between 2 layers of collagen, a sandwich configuration, remained viable and sustained liver-specific function for prolonged periods of time. In addition, hepatocytes maintained in the collagen sandwich configuration displayed a distribution of actin filaments similar to that

observed *in vivo*. Furthermore, LeCluyse *et al.* (1994) demonstrated that sandwich-cultured hepatocytes sustained normal cell polarity and formed extensive bile canaliculi. Recently, it was demonstrated that sandwich-cultured hepatocytes maintained bile acid synthesis (3 α -hydroxysteroid dehydrogenase) and cytochrome P450 enzyme induction potential (LeCluyse *et al.*, 1996). In hepatocytes cultured conventionally, phase II enzymatic activity is maintained over that of phase I. The cytochrome P450-dependent monooxygenases, in particular, are readily lost (Rogiers and Vercruysse, 1993). Niemann *et al.* (1991) reported that there was a reduction in relative enzyme activities in both phase I and phase II metabolism, suggesting a shift towards a more de-differentiated drug metabolism pattern. Although phase I enzymatic activity decreases in both sandwich-cultured hepatocytes and conventionally cultured hepatocytes, the sandwich-cultured hepatocytes exhibit superior cytochrome P450 inducibility (LeCluyse *et al.*, 1996). Sandwich-cultured hepatocytes form intact canalicular networks, allowing a readily accessible compartment for quantitation of substances excreted into bile. This novel *in vitro* model system offers a distinct advantage over existing methodology and some exciting possibilities for examining canalicular transport processes and mechanisms of hepatobiliary disposition. However, the transport function on both sinusoidal and canalicular membrane domains remains to be characterized before this *in vitro* model can be utilized for transport studies.

Tight Junctions

Hepatocytes are polarized epithelial cells with two membrane domains, basolateral (sinusoidal) and apical (canalicular). Like other epithelia, a junctional complex continuously delineates the apical from the basolateral portions of the cell surface. The cell-cell junctional complexes are composed of three parts: zonula occludens (tight junctions), zonula adherens (intermediate junctions) and macula adherens (desmosomes) (Madara *et al.*, 1978). Tight junctions provide a continuous seal around the apical region of the lateral membranes of adjoining epithelial cells, preventing the free passage of large molecules and ions through the paracellular pathway. This barrier function of tight junctions distinguishes them from other types of junctions.

Cumulative evidence indicates that tight junction structure and function can be altered by a variety of physiologic and nonphysiologic stimuli. Among the nonphysiologic stimuli, the most thoroughly studied is low extracellular calcium concentrations. Depletion of extracellular calcium ions with chelating agents or low concentrations of calcium in the medium causes junctional splitting or disassembly in a variety of epithelial models (Meldolesi *et al.*, 1978, Kartenbeck *et al.*, 1991, Meza *et al.*, 1980). When epithelial cell cultures are transferred from a medium with a normal extracellular calcium concentration (1-2 mM) to a medium with a low extracellular calcium concentration (< 0.05 mM free Ca^{2+}), cell-cell contacts are disrupted. For instance, in Madin-Darby canine kidney cells, calcium removal disrupts junctional integrity within 5 minutes. Disruption of junctional integrity under low calcium concentrations may not affect directly the tight junction, but may alter the extracellular membrane domain of tight junction complexes, which subsequently transduces

signals to the components of the cytoplasmic plaque domain. The exact mechanism of signal transduction still remains to be elucidated. Protein kinases appear to be involved in this process, since protein kinase inhibitors, *e.g.*, H-7 (a protein kinase C inhibitor), can block disruption of tight junctions caused by extracellular Ca^{2+} depletion (Citi, 1992).

Maintenance of extracellular Ca^{2+} concentrations within the normal physiological range (1-2 mM) is essential *in vivo* to sustain the barrier function of tight junctions and to prevent movement of actively secreted bile acids from bile into blood. Previous studies have shown that bile flow and biliary taurocholate excretion in isolated perfused rat livers decrease, and permeability of the paracellular pathway increases significantly, at low extracellular Ca^{2+} concentrations (at or below 0.05 mM; Anwer and Clayton, 1985; Graf, 1976). Similar results also were observed in 96-hour sandwich-cultured hepatocytes. After brief treatment of 96-hour sandwich-cultured hepatocytes with 0.1-0.2 mM EDTA, the carboxyfluorescein concentrated in the canalicular networks was released presumably because of disruption of the tight junctions that seal the canaliculi (LeCluyse *et al.*, 1994). The increase in paracellular permeability allows regurgitation of secreted substrate and thereby decreases biliary excretion.

Freshly isolated hepatocytes retain their original polarity, *e.g.*, each single cell is surrounded by a band of canalicular cell membrane (Graf and Boyer, 1990). Within the first 2 hours of tissue culture, canalicular membranes are almost completely removed from the cell surface except for retained cell-cell contact zones. At the site of cell-cell adhesion, tight junction-associated protein ZO-1 and canalicular marker enzyme Mg^{2+} -ATPase have been identified by immunofluorescence staining in short-term (4-8 hours) cultured hepatocyte

couplets, suggesting the existence of bile canalicular-like structures (Stevenson *et al.*, 1988, Graf and Boyer, 1990). In long-term (2-7 days) cultured hepatocytes in a sandwich configuration, a junctional complex-like structure and canalicular marker enzymes (Mg²⁺-ATPase, dipeptidyl peptidase and aminopeptidase N) have been reported at the canalicular space (LeCluyse *et al.*, 1994). Recently, Talamini *et al.* (1997) demonstrated the existence of junctional protein, uvomorulin (E-cadherin), in hepatocytes cultured in a sandwich configuration.

The functional integrity of tight junctions can be assessed directly by localization of ruthenium red staining with electron microscopy. Ruthenium red is a crystalline inorganic hexavalent cation with a diameter of approximately 1.13 nm that reacts with, and binds to, polyanionic molecules including mucopolysaccharides, glycosaminoglycans and certain lipids found on the cell surface (Hayat, 1993). Ruthenium red does not penetrate intact plasma membranes, but it binds to intercellular membranes, and penetrates to the level of the tight junction in non-leaky epithelia (Mullin *et al.*, 1997; van Deurs *et al.*, 1996). Recently, by using ultrastructural analysis with ruthenium red, Talamini *et al.* (1997) demonstrated complete penetration of the marker ruthenium red between cell membranes in rat hepatocytes cultured for 24 hours, showing that tight junctions have not yet developed. After the hepatocytes were cultured in a sandwich configuration for 120 hours, ultrastructural analysis revealed exclusion of ruthenium red, indicating that tight junctions were formed in the long-term sandwich-cultured hepatocytes.

Hypothesis

Numerous *in vivo* and *in vitro* preparations have been used to investigate biliary excretion processes. However, these existing methods may not always be applied to investigate human biliary excretion. In addition, current approaches cannot be used to examine efficiently biliary excretion processes for a large number of compounds in the drug discovery and development process. Therefore, there is a need for a rapid and inexpensive *in vitro* screening method that is predictive of hepatobiliary disposition in animals and humans, especially in this modern era of high synthetic capabilities for drug candidates (e.g., combinatorial chemistry approaches). Hepatocytes cultured between 2 layers of collagen gel in a sandwich configuration represent a promising *in vitro* model to meet this need.

Previous studies have demonstrated that long-term primary rat hepatocytes cultured in a sandwich configuration maintain normal morphology, develop extensive canalicular networks, and sustain liver-specific functions (Dunn *et al.*, 1989, LeCluyse *et al.*, 1996). These long-term sandwich-cultured hepatocytes represent a potential *in vitro* model to study biliary excretion. The hypothesis underlying this dissertation research was that sandwich-cultured primary rat hepatocytes restore hepatobiliary membrane transport activities and thus can be utilized to study biliary excretion.

In order to examine the effects of extracellular matrix on the maintenance of sinusoidal membrane transport systems, the expression and functional activity of Ntcp in hepatocytes cultured under conventional conditions or in a sandwich configuration were assessed by immunoblot analysis of Ntcp and by examination of the uptake kinetics of the Ntcp substrate taurocholate (Chapter 2). In order to examine the canalicular membrane

transport systems in long-term sandwich-cultured hepatocytes, the expression and function of cMOAT were evaluated by immunoblot analysis of cMOAT and localization of the cMOAT substrate carboxydichlorofluorescein (Chapter 3). Kitamura *et al.* (1990) demonstrated that carboxydichlorofluorescein is a cMOAT substrate. Biliary excretion of this compound is negligible in TR⁻ rats. In the present study, carboxydichlorofluorescein was employed as a model substrate to examine the functional activity of cMOAT. Carboxydichlorofluorescein diacetate, which exhibits only weak fluorescence, was utilized due to its rapid penetration into the hepatocyte plasma membrane. Carboxydichlorofluorescein diacetate is hydrolyzed readily in the cytoplasm by intracellular esterases to a highly fluorescent product, carboxydichlorofluorescein (Haugland, 1992).

The amount of substrate excreted into the canalicular networks of sandwich-cultured hepatocytes, *i.e.*, the extent of biliary excretion in the *in vitro* model, was quantitated by the differential cumulative uptake of substrates in the hepatocyte monolayers pre-incubated in standard buffer and Ca²⁺-free buffer (Chapter 3). A multiexperimental approach was used to examine the three hypotheses involved in this novel quantitative method. (1) Ca²⁺ depletion disrupts the barrier function of tight junctions between the canalicular lumen and the extracellular space (Chapter 4). This hypothesis was tested by determining the localization of ruthenium red (an electron microscopic extracellular marker) staining in monolayers incubated in standard buffer or Ca²⁺-free buffer utilizing electron microscopy. (2) Substrate in the canalicular space and in the extracellular space diffuses rapidly via a paracellular pathway during Ca²⁺ depletion (Chapter 3). This hypothesis was tested by determining the localization of carboxydichlorofluorescein (a canalicular space marker) and rhodamine-

dextran (an extracellular space marker) in the monolayers incubated in standard buffer or Ca^{2+} -free buffer utilizing confocal fluorescence microscopy. (3) Ca^{2+} depletion does not change membrane transport properties (Chapter 4). This hypothesis was tested by examining the cumulative uptake of taurocholate in freshly isolated hepatocytes, which lack intact canaliculi, in standard buffer or Ca^{2+} -free buffer. The re-establishment of functional polarity in sandwich-cultured hepatocytes was examined (Chapter 3). This study was designed to assess quantitatively the time course of re-establishment of functional polarity in the cultured hepatocytes. In addition, the disposition of the model substrate taurocholate was evaluated in long-term sandwich-cultured hepatocytes pre-incubated in standard buffer or Ca^{2+} -free buffer (Chapter 4). This study was designed to test the hypothesis that long-term sandwich-cultured hepatocytes can be used to study hepatobiliary disposition. Finally, the correlation between biliary excretion in long-term sandwich-cultured hepatocytes and *in vivo* was assessed (Chapter 5). This study was designed to test the hypothesis that cultured hepatocytes can be employed to study and to predict *in vivo* biliary excretion.

In summary, hepatobiliary transport is an important component of hepatic disposition processes. The membrane transport systems for biliary excretion have been examined with a variety of techniques. However, no technique is able to investigate efficiently biliary excretion in animals and humans. This dissertation research demonstrates that long-term sandwich-cultured hepatocytes can be used as an *in vitro* model to study biliary excretion. It is hoped that development of this *in vitro* model will facilitate the investigation of biliary excretion of endogenous and exogenous compounds. This *in vitro* model may be used to predict biliary excretion for new drug candidates, and to elucidate the underlying

mechanisms of biliary excretion. In addition, techniques developed in this dissertation
— research can be employed directly to establish a similar *in vitro* model to study human
hepatobiliary disposition.

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CHAPTER 2

PARTIAL MAINTENANCE OF TAUROCHOLATE UPTAKE BY ADULT RAT HEPATOCYTES CULTURED IN A COLLAGEN SANDWICH CONFIGURATION

This chapter has been submitted to the *Pharmaceutical Research* (*accepted, pending revision*), and is presented in the style of that journal.

ABSTRACT

Purpose. This study was designed to characterize taurocholate uptake properties in primary cultures of rat hepatocytes maintained under different matrix conditions. **Methods.** Hepatocytes isolated from male Wistar rats (230-280 g) were cultured on a simple collagen film, on a substratum of gelled collagen or between two layers of gelled collagen (sandwich configuration). Hepatocyte morphology, taurocholate uptake properties, and expression of the sinusoidal transport protein, Na^+ /taurocholate-cotransporting polypeptide (Ntcp) were examined in these cultures at day 0 and day 5. **Results.** By day 5, monolayer integrity had deteriorated in simple collagen cultures. In contrast, cell morphology was preserved in hepatocytes maintained in a sandwich configuration. At day 5, taurocholate accumulation at 5 min in hepatocytes cultured on a simple collagen film, on a substratum of gelled collagen, and in a sandwich configuration was ~13%, 20% and 35% of day-0 levels, respectively, and occurred predominately by a Na^+ -dependent mechanism. The initial taurocholate uptake rate vs. concentration (1-200 μM) profile was best described by a combined Michaelis-Menten and first-order function. In all cases, the estimated apparent K_m values were comparable for day-0 and day-5 hepatocytes (32-41 μM). In contrast, the V_{\max} values of hepatocytes cultured on a simple collagen film, on gelled collagen and in a sandwich configuration were ~5, 6 and 14% of the values at day 0, respectively; values for the first-order rate constant were 5-, 3- and 2-fold lower, respectively. Immunoblot analysis indicated that at day 5 Ntcp expression in hepatocytes cultured in a sandwich configuration was greater than in hepatocytes cultured on a simple collagen film. **Conclusions.** A collagen sandwich configuration reestablishes normal

morphology and partially restores bile acid uptake properties in primary cultures of rat hepatocytes.

INTRODUCTION

Primary cultures of hepatocytes currently are used in drug discovery and development to evaluate hepatic transport and metabolism of drugs, cytochrome P450 induction, drug interactions and drug-associated hepatotoxicity (1). However, long-term cultured hepatocytes have not been used to study hepatobiliary transport due to a rapid loss of transport activities (2-5). For instance, the maximal taurocholate uptake capacity (i.e., V_{\max}) was reduced to ~4% of day-0 levels in primary cultures of rat hepatocytes maintained for 72 hours under conventional culture conditions (6, 7). Inasmuch as Na^+ -dependent bile acid uptake represents a typical differentiated hepatocyte function, these observations agree well with the general concept that, under conventional culture conditions, primary cultures of rat hepatocytes dramatically lose many liver-specific properties and, therefore, are not a good model to study hepatobiliary transport (8, 9).

This trend in the "de-differentiation" of cultured hepatocytes appears to be delayed by altering the biophysical state of the extracellular matrix. Several studies have shown that hepatocytes respond to a substratum composed of gelled collagen or Matrigel by expressing more liver-specific functions including bile acid synthesis while reducing the over-expression of "common genes" (e.g., actin) (10, 11). Moreover, hepatocytes cultured between two layers of gelled collagen possess superior morphological and biochemical properties, such as albumin secretion, bile acid synthesis, and P450 enzyme induction, compared to cells on a single layer of gelled collagen (12-14). In particular, collagen-sandwiched hepatocytes develop an elaborate network of bile canaliculi that exhibit many normal structural and functional properties (13). Preliminary studies suggested that the measurement of substrates localized in

the canalicular spaces may be utilized to predict *in vivo* biliary excretion of those substrates (15). However, a careful investigation of the effects of varying the geometry of the extracellular matrix on the retention of the bile acid transporters in primary cultures of rat hepatocytes has not been reported.

In this study, bile acid transport properties were characterized in primary cultures of rat hepatocytes maintained under different matrix conditions for up to 5 days. Taurocholate, a well-documented substrate of bile acid transporters, was employed as a model compound. Taurocholate enters hepatocytes predominantly through the Na^+ /taurocholate-cotransporting polypeptide (Ntcp) and to a lesser extent by a Na^+ /independent organic anion transporter (16).

Total cumulative taurocholate uptake, initial taurocholate uptake rate and cell morphology were assessed in 5-day-old cultures of hepatocytes maintained either on a simple collagen substratum (rigid collagen), on a single layer of gelled collagen or between two layers of gelled collagen (sandwich configuration). The expression of Ntcp in hepatocytes cultured on rigid collagen or in a sandwich configuration was assessed by immunoblot analysis.

MATERIALS AND METHODS

Chemicals. Taurocholate and dexamethasone were obtained from Sigma Chemical Co. (St. Louis, MO). [^3H]Taurocholic acid (3.4 Ci/mmol) was purchased from DuPont New England Nuclear (Boston, MA). Collagenase was obtained from Worthington Biochemical Corp. (Freehold, NJ). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum and insulin were purchased from Gibco (Grand Island, NY). Rat tail collagen (type I) was obtained from Collaborative Biomedical Research (Bedford, MA). All other chemicals and reagents were of analytical grade and were readily available from commercial sources.

Animals. Male Wistar rats (250-280 g) from Charles River (Raleigh, NC) were used as liver donors. They were allowed free access to food and water, and were housed in a constant alternating 12-hr light and dark cycle. All procedures were approved by the Institutional Animal Care and Use Committee.

Preparation of Culture Dishes. Plastic culture dishes (60 mm) were precoated with collagen, type I (0.1 ml, 1.5 mg/ml), in either a gelled or rigid state at least 1 day prior to preparing the hepatocytes (14). To obtain a simple (rigid) substratum, collagen solution was added to each dish and spread evenly with a Teflon policeman. Coated dishes were stored in a sterile hood overnight. Immediately before use, fresh medium was added to neutralize the collagen. To obtain a gelled substratum, neutralized collagen (0.1 ml, 1.5 mg/ml) was prepared and spread onto petri dishes as described above. Freshly coated dishes were placed at 37°C in a humidified incubator for at least 60 min to allow the matrix material to gel, followed by addition of 3-ml fresh medium to the dishes and storage in a humidified incubator.

Isolation and Culture of Rat Hepatocytes. Hepatocytes were isolated from rats with a two-step perfusion modification of previously described methods (14). Briefly, the liver was perfused *in situ* with oxygenated calcium-free Krebs-Henseleit bicarbonate buffer containing 5.5 mM glucose for ~10 min at 37°C followed by perfusion with buffer containing 1 mM calcium and collagenase type I (0.5 mg/ml) for 10 min. After collagenase digestion, released cells were filtered through a sterile nylon 70- μ m filter into two 50-ml centrifuge tubes and washed once with Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum, insulin (4 mg/L), and dexamethasone (1 μ M). The cell pellet (50 \times g, 3 min) was resuspended in fresh DMEM and an equal volume of 90% isotonic Percoll; the resulting cell suspension was centrifuged at 150 \times g for 5 min. Hepatocyte viability was determined by trypan blue exclusion and was typically >90%. Cells were diluted with fully-supplemented medium and 2-ml aliquots were added to precoated dishes at a density of approximately 2×10^6 cells/60-mm dish. Approximately 1 hr after plating the cells, the medium was aspirated and replaced with fresh fully-supplemented DMEM. For hepatic transport studies, hepatocytes that had been seeded for 2-4 hr without collagen overlay were defined as day-0 or short-term cultured hepatocytes. To prepare cultures in a sandwich configuration, an ice-cold neutralized collagen solution (0.1 ml) was added to the top of cultures 24 hr after plating (14). These cultures were incubated 1 hour at 37°C to allow collagen to gel before adding 3-ml fresh medium to the dishes. Medium was changed on a daily basis until uptake studies were performed on the fifth day. These hepatocytes were referred to as day-5 or long-term cultured hepatocytes.

Taurocholate Uptake Studies. To validate the methods for assaying taurocholate uptake in cultures of primary rat hepatocytes maintained in various matrix geometries, experiments were performed in cell-free culture dishes coated with gelled collagen with a collagen overlay, or hepatocytes cultured in a collagen sandwich configuration. Under these conditions, the influence of the matrix on background or non-specific binding by the tracer compound was determined after a series of washes with Hanks' balanced salt solution (HBSS). Dishes with a double-gel (without hepatocytes) or cultures in a sandwich configuration (with hepatocytes) were incubated for 10 min in 3 ml of 1 μ M [3 H]taurocholate in HBSS followed by sequential washes with 3-ml ice-cold HBSS. Each wash step lasted for approximately 10 seconds. Cells cultured in a sandwich configuration were lysed with 3-ml water after rinsing with 3-ml HBSS 9 times; thereafter washing with 3-ml HBSS was resumed.

Cultures were rinsed 2 times with 3-ml HBSS to remove incubation medium prior to transport studies. Each 60-mm dish received 3 ml of HBSS or choline buffer, in which sodium chloride was replaced with choline chloride on an equal molar basis, and was placed at either 4° or 37°C for 10 min before initiating experiments. Uptake was initiated by adding HBSS or choline buffer containing [3 H]taurocholate to each dish at designated concentrations (see RESULTS). Incubations were carried out at 4 or 37°C for designated times. Taurocholate uptake was terminated by washing the cultures 4 times with ice-cold HBSS. After washing, 2 ml of 1% Triton X-100 solution was added to the culture dishes to lyse cells and an aliquot of lysate was analyzed by liquid scintillation spectrometry. Protein was measured with Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). Data in the plots are expressed as mean \pm SD from 3 or 4 separate preparations of hepatocytes; triplicates were performed in

each preparation, unless indicated otherwise. All values for taurocholate uptake into cell monolayers were corrected for nonspecific binding by subtracting taurocholate uptake determined in the appropriate control dishes in the absence of cells.

Immunoblot analysis of Ntcp. Hepatocyte cultures were rinsed 1 time with 3-ml ice-cold HBSS. Cells were collected by scraping into hypotonic lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 0.4 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, and 10 μ g/ml leupeptin) and incubated in an ice bath for 15 min. The swollen cells were disrupted with 30 strokes in a tightly fitting Dounce homogenizer. The homogenate was centrifuged at 400 \times g for 10 min at 4°C. The supernatant was centrifuged at 30,000 \times g for 30 min at 4°C and the resulting pellet was used as the crude membrane fraction (18). Proteins from crude membrane fractions (50 μ g) were subjected to 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA) electrophoresis by the method of Laemmli (19). After proteins were transferred electrophoretically from SDS gels to nitrocellulose membranes (0.45 μ m), the blots were blocked with Tris-buffered saline containing 0.05% Tween 20 and 5% non-fat dry milk for 1 hr at room temperature. Rabbit anti-serum raised against a fusion protein containing the maltose-binding protein and the carboxyl-terminus of Ntcp was used as the primary antibody in the immunoblot analysis in the current studies. The specificity of this anti-serum has been demonstrated previously (17). The blots were probed with polyclonal anti-Ntcp rabbit serum at 1:4000 dilution. Antibody binding was visualized with horseradish peroxidase-conjugated donkey anti-rabbit Immunoglobuline G serum at 1:2000 dilution, followed by detection with Amersham

enhanced chemiluminescence kit and exposure on Amersham Hyperfilm according to the manufacturer's instructions.

Data analysis. A combined Michaelis-Menten (K_m and V_{max}) and first-order function (K) was fit to the average of the initial rate of taurocholate uptake vs. concentration data to obtain estimates of the relevant kinetic parameters utilizing a nonlinear least-squares regression method (PCNONLIN, Version 3.0, SCI Software, Statistical Consultants, Inc., Lexington, KY). The criteria used to obtain the best model included Akaike's Information Criterion (20), the degree of co-linearity of parameters, the degree of bias in residual error, and visual inspection of the generated curves relative to the data. ANOVA followed by Duncan's multiple range test was employed to test the significance of the effect of culture conditions on taurocholate uptake with $p < 0.05$ considered statistically significant.

RESULTS

— In order to examine accurately the kinetics of bile acid transport, it was essential to develop a wash procedure to remove most of the extracellular substrate efficiently after transport studies while leaving the intracellular substrate intact. Figure 2-1 represents the remaining taurocholate in a cell-free double-layer collagen gel and in hepatocyte cultures in a collagen sandwich configuration after each wash. During the first 4 washes, more than 99.8% of taurocholate was removed from the double-layer gel; 4 additional washes removed less than 0.2% of the original substrate. In collagen-sandwich cultures, the first 4 washes removed 74.0% of taurocholate; the next 4 washes only removed 0.7% of the original taurocholate. After the cells were lysed with water, 25.1% of taurocholate was removed by 4 washes and less than 0.2% of taurocholate remained in the cultures. These results indicated that 4 washes were adequate to remove most of the extracellular substrate while retaining the intracellular substrate. In addition, the nonspecific binding of taurocholate onto the hepatocyte plasma membrane was negligible because the residual taurocholate in the double-layer gel and in the lysed cells cultured in a collagen-sandwich configuration was similar after 4 washes. In the present study, the adapted wash procedure for 60 mm petri dishes was 4 washes with 3-ml ice-cold buffer for approximately 10 seconds each.

Primary cultures of rat hepatocytes were maintained for 2-4 hours on either a layer of gelled collagen (Figure 2-2A) or on a rigid collagen substratum (Figure 2-2B) before conducting transport studies. Regardless of the substratum, hepatocytes began to show signs of cell spreading almost immediately after cell attachment. Hepatocytes maintained on a gelled collagen substratum displayed more compact spheroidal morphologies (Figure 2-2A). In

contrast, hepatocytes maintained on a rigid collagen substratum spread and flattened more extensively (Figure 2-2B).

[³H]Taurocholate accumulation over time measured in the hepatocytes cultured on a rigid and gelled collagen for 2-4 hr is represented in Figure 2-3A and B, respectively. Considerable differences were observed between the passive (4°C) and active (37°C) components of taurocholate uptake for both matrix conditions. The non-specific, passive diffusion (4°C) was less than 10% of the uptake at 37°C in both matrix conditions. Total taurocholate uptake in day-0 hepatocytes at 37°C was significantly higher in the cells grown on gelled collagen compared to rigid collagen. Taurocholate uptake was reduced by 70-80% in Na⁺-free buffer in the hepatocytes maintained on a rigid or gelled collagen substratum but the uptake was higher than the simple diffusion process (4°C; $p < 0.05$).

The second portion of this study was designed to examine taurocholate uptake properties in primary cultures of rat hepatocytes maintained on a rigid collagen substratum, on a gelled collagen substratum, or in a sandwich configuration for extended periods of time (5 days). Hepatocytes cultured on a rigid film continued to spread until a confluent monolayer was formed (Figure 2-2D). Although cultures of hepatocytes which had been maintained on gelled collagen for 5 days showed regions where the cells had reached confluence as was observed in cultures grown on a rigid film, cells on the gelled collagen remained in a more 3-dimensional state (Figure 2-2C). Under both matrix conditions without an overlay of collagen, hepatocytes typically exhibited significant spreading, a deteriorated morphology, and few signs of intact bile canaliculi networks. In contrast, hepatocyte cultures maintained in a sandwich configuration remained in clusters or chords and exhibited less cell spreading compared to the

other cultures with open spaces between clusters of cells (Figure 2-2E). In addition, hepatocytes cultured in a sandwich configuration displayed a more compact, 3-dimensional morphology and prominent networks of bile canaliculi throughout the cultures.

Significant [^3H]taurocholate uptake activity was maintained in hepatocytes cultured in a sandwich configuration at day 5 (Figure 2-3). The mean values of [^3H]taurocholate at day-5 were greatest in the hepatocytes cultured in a sandwich configuration compared to on a rigid or gelled collagen substratum (Figure 2-3C-E; $p < 0.05$). For instance, on average at 5 min, hepatocytes cultured in a sandwich configuration retained ~35% of the original taurocholate uptake capacity of day-0 cultures while hepatocytes cultured on rigid and gelled collagen retained ~13 and 20%, respectively. The simple diffusion of [^3H]taurocholate at 4°C was not significantly different among these cultures. Although the uptake of 1 μM [^3H]taurocholate was reduced extensively in Na^+ -free buffer in collagen-sandwich cultures at day 5, it was still greater than the uptake at 4°C ($p < 0.05$).

Kinetic parameters describing taurocholate uptake were determined in hepatocytes cultured for 2-4 hr and hepatocytes cultured for 5 days on a rigid, or a gelled collagen substratum, or in a sandwich configuration. Taurocholate uptake vs. time appeared to be linear during the initial 60 seconds for all concentrations except 200 μM (Figure 2-4). Based on these results, initial rates of taurocholate uptake were determined between 15-30 seconds. A plot of the average initial rates of taurocholate uptake vs. substrate concentration (1-200 μM) from all culture configurations is illustrated in Figure 2-5.

Three kinetic models, first-order function, Michaelis-Menten process and Michaelis-Menten process in parallel with first-order function, were examined to select the best model

to described the initial uptake rates. Based on model selection criteria, a combined Michaelis-Menten and first-order function best described the initial uptake data using nonlinear least-squares regression analysis. Dissociation constants (K_m) and maximal rates of uptake (V_{max}) for the nonlinear uptake component and the first-order rate constant (K) for the linear uptake component were estimated from the mean data in Figure 2-5. Estimates of K_m were comparable for day-0 and day-5 cultures regardless of the matrix configuration and ranged from 32-41 μ M (Table 2-1). In contrast, day-5 hepatocytes cultured in a collagen-sandwich cultures exhibited the greatest V_{max} value compared to hepatocytes cultured on rigid or gelled collagen for 5 days. The V_{max} of the hepatocytes cultured on rigid collagen, gelled collagen and in a sandwich configuration at day 5 was ~5, 6 and 14% of the values at day 0, respectively. In addition, sandwich configuration and gelled collagen conditions resulted in 2- to 3-fold lower first-order rate constant values for the linear portion of taurocholate uptake in day-5 cultures compared to that exhibited in day-0 cultures; however rigid collagen conditions resulted in a 5-fold lower rate for the linear portion of taurocholate uptake.

Immunoblot analysis showed that similar levels of Ntcp immunoreactive protein (51 kDa) are expressed in rat hepatocytes cultured on either a gelled or a rigid substratum for 2-4 hours (Figure 2-6, lanes 1 and 3). At day-5, Ntcp immunoreactive protein was detected in the hepatocytes cultured in a sandwich configuration (Figure 2-6, lane 2) but was undetectable in hepatocytes cultured on a rigid substratum (Figure 2-6, lane 4). After enhancement of the sensitivity by an extended exposure time of the blot, the band intensity at 51 kDa increased significantly in the hepatocytes maintained in a sandwich configuration; however, Ntcp

immunoreactive protein was not detectable in the hepatocytes cultured on a rigid collagen substratum (blots not shown).

DISCUSSION

The morphology of hepatocytes maintained in culture was drastically affected by the biophysical state of the extracellular matrix environment. At 2-4 hours, hepatocytes exhibit more spreading on rigid collagen compared to on gelled collagen. The most prominent difference between rigid collagen, gelled collagen and collagen-sandwich cultures was overall cell shape and morphology after cells were cultured for 5 days. Hepatocytes maintained on the rigid collagen dishes for 5 days were more prone to spread and flatten. Electron micrographs of similar preparations showed that these cultures retain few of the morphological features of their native counterparts (13). On the other hand, hepatocytes maintained on a gelled substratum of collagen (with or without a top layer of collagen) retained a more compact, three-dimensional shape and remained clustered in cell chords or trabeculae. Hepatocytes cultured in a collagen sandwich configuration displayed less cell spreading compared to rigid collagen and showed prominent networks of bile canaliculi throughout the culture period. This architectural conformation more closely resembled the cell plates, sinusoidal spaces and bile canaliculi observed in the intact liver.

Similarly, cumulative taurocholate uptake of hepatocytes maintained in culture also was affected significantly by the extracellular matrix environment. Taurocholate accumulation in hepatocytes cultured for a few hours on gelled collagen was greater compared to the cells on rigid collagen at 37°C while no difference was observed at 4°C. These observations indicated that the active transport process is maintained better in hepatocytes cultured on gelled collagen, but simple diffusion was essentially the same. These data confirm previously reported results that transport capacity of cultured hepatocytes deteriorates rapidly under conventional culture

conditions (2). Taurocholate accumulation deteriorated with time in hepatocytes maintained in culture for up to 5 days. However, this decrease in transport capacity was attenuated by the addition of a collagen gel substratum and overlay. Collagen-sandwich cultures at day 5 retained as much as 35% of the cumulative uptake (5 min) of day-0 cultures. Further studies have indicated that cumulative uptake of taurocholate in sandwich-cultured hepatocytes decreases gradually. After the hepatocytes were maintained in a sandwich configuration for 72 hr, cumulative taurocholate uptake was 63% of the uptake in the hepatocytes cultured for 2-4 hr. In contrast, previous studies reported that hepatocytes maintained under conventional conditions only retain 2-10% of cumulative uptake at 72 hr (2, 3). Thus, primary rat hepatocytes cultured in a collagen-sandwich configuration retain significant taurocholate cumulative uptake for a prolonged period of time.

Maintenance of the expression of transport proteins such as Ntcp in long-term cultured hepatocytes was affected significantly by the extracellular matrix. Western blot analysis demonstrated the presence of immunoreactive Ntcp protein (molecular mass 51 kDa) in crude membranes isolated from cultured hepatocytes. After prolonged exposure, the intensity of the Ntcp band was enhanced markedly in hepatocytes cultured in a sandwich configuration, but could not be detected in hepatocytes cultured on a rigid collagen substratum. These results show that Ntcp was maintained partially in the hepatocytes cultured in a collagen-sandwich configuration but was lost completely in hepatocytes cultured on a rigid collagen substratum at day 5. These differences in taurocholate uptake properties observed between hepatocytes cultured under different extracellular matrix conditions may be attributed to greater levels of

Ntcp expression in hepatocytes cultured in collagen-sandwich configuration compared to hepatocytes cultured on rigid gelled collagen.

Liang, *et al* (2), used a specific cDNA probe of the liver Ntcp to quantitate the relative abundance of the specific cotransporter mRNA in rat hepatocytes maintained in primary cultures for up to 72 hours. Their results demonstrated a parallel reduction of the Na^+ -dependent taurocholate uptake capacity and the Ntcp-encoding mRNA levels with increasing culture times. Hence, down regulation of the Ntcp gene appears to be the primary cause of incompetent Na^+ -dependent bile acid uptake in cultured rat hepatocytes. In this study, hepatocytes cultured in a collagen-sandwich configuration maintained taurocholate uptake properties and Ntcp, suggesting that the collagen-sandwich configuration may be able to restore partially Ntcp gene expression and/or to stabilize Ntcp protein.

Taurocholate uptake in collagen-sandwich cultures occurred mainly by a Na^+ -dependent mechanism and to a lesser extent by a Na^+ -independent mechanism in both hepatocytes cultured for 2-4 hours on gelled collagen and for 5 days in a sandwich configuration. These results are consistent with published data indicating that taurocholate uptake *in vivo* occurs predominantly by a Na^+ -dependent mechanism (16). Cultured hepatocytes treated with Na^+ -free buffer showed a 70-80% reduction at day 0 but 90-95% reduction at day 5 in the total cumulative taurocholate uptake compared to untreated cultures. These results suggest that the Na^+ -independent component of taurocholate uptake deteriorates more rapidly than the Na^+ -dependent component.

The mean K_m values for hepatocytes cultured under all conditions were similar, however, estimates for V_{\max} in hepatocytes cultured on rigid collagen, gelled collagen and in a

sandwich configuration at day 5 were 5, 6 and 14% of the values at day-0, respectively. These findings are in agreement with reports for taurocholate uptake in hepatocytes indicating that K_m values remain relatively stable over time in culture, but V_{max} progressively declines (2). In sandwich-cultured hepatocytes, the V_{max} for initial uptake and the 5-min cumulative uptake were 14% and 35% of the respective values in 2-4 hr cultured hepatocytes. This apparent discrepancy is probably due to the absence versus presence of a bile canalicular compartment in short-term and long-term cultured hepatocytes, respectively, resulting in greater apparent cumulative uptake capacity in long-term cultured hepatocytes. Interestingly, the first-order rate constant for taurocholate uptake at day 5 was 5-, 3- and 2-fold lower than the values at day 0. It has yet to be determined whether the reduction in the rate constant for the linear portion of taurocholate uptake in hepatocytes cultured under different extracellular matrix conditions reflects deterioration in one of the taurocholate transporters (i.e., Na^+ -independent) or the inability of one transporter to function effectively.

Inasmuch as hepatocytes cultured in a sandwich configuration form extensive bile canalicular networks and partially maintain bile acid uptake function, this *in vitro* model may have several applications in drug discovery and development. For instance, preliminary studies suggested that endogenous and exogenous compounds can accumulate in the canalicular networks of primary hepatocytes cultured in a sandwich configuration for 5 days. Canalicular substrate accumulation may be quantitated by modulating Ca^{2+} in the incubation buffers (15). Thus, as Caco-2 cell monolayers are used to study drug absorption properties, this *in vitro* model also may have utility as a screening tool to identify the extent of biliary excretion of drug candidates *in vivo*.

— In summary, this study has demonstrated that hepatocytes cultured in a collagen sandwich configuration retain superior bile acid transport properties over time compared to hepatocytes cultured without a collagen overlay. Hepatocytes cultured in a sandwich configuration may be a useful model for *in vitro* studies of the expression and regulation of hepatobiliary transport function.

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Table 1. Kinetic Parameters of Taurocholate Uptake in Cultured Hepatocytes

Culture Condition	K_m^*	V_{max}^*	K^*
	(μ M)	($\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$)	($10^{-3} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$)
Gelled Collagen (day 0)	36.9 ± 7.4	5.20 ± 0.39	3.85 ± 0.34
Sandwich Configuration (day 5)	35.8 ± 7.6	0.716 ± 0.056	1.69 ± 0.05
Gelled Collagen (day 5)	32.2 ± 15.9	0.291 ± 0.052	1.19 ± 0.05
Rigid Collagen (day 5)	40.6 ± 27.1	0.259 ± 0.061	0.74 ± 0.05

* Parameters (mean \pm SE, n = 6-9) were obtained by fitting a Michaelis-Menten and a first-order function to the data in Figure 2-5 with nonlinear least-squares regression analysis.

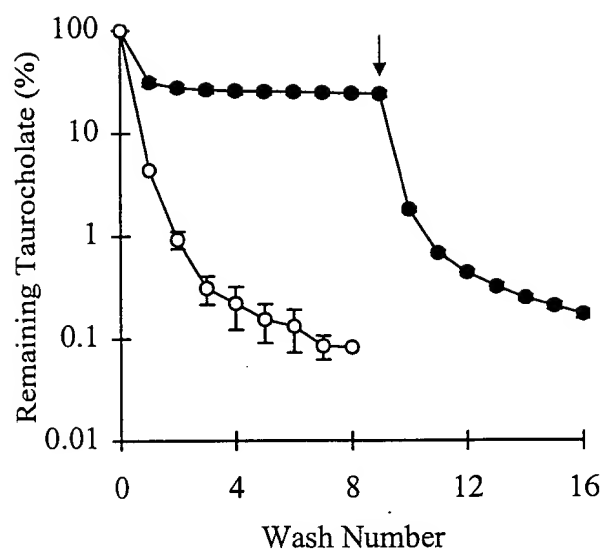


Figure 2-1. Percent of radioisotope remaining vs. wash number in petri dishes containing a double-layer of collagen gel without hepatocytes (O) or a double-layer of collagen gel with hepatocytes (●). Dishes were incubated for 10 min in 3 ml of 1 μ M [3 H]taurocholate in HBSS followed by sequential washes with 3-ml HBSS. At the ninth wash (arrow), 3, ml of water was added to collagen sandwich cultures to lyse the cells; thereafter washing with 3-ml HBSS was resumed.

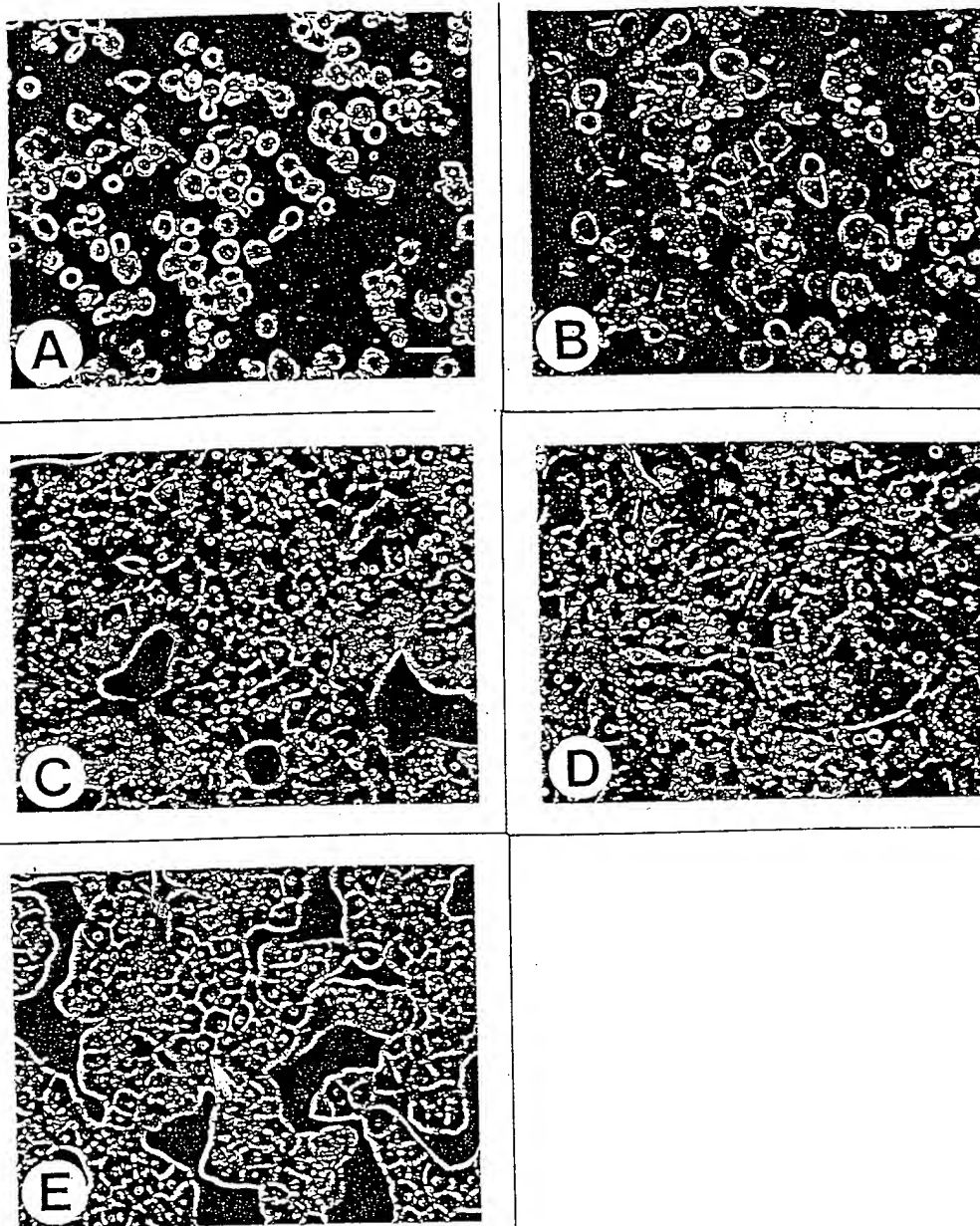


Figure 2-2. Effects of extracellular matrix configuration on hepatocyte morphology. Hepatocyte cultures maintained for either 2-4 hr (A, B) or 5 days (C, D & E) on a rigid collagen substratum (B, D), a gelled collagen substratum (A, C), or between two layers of gelled collagen (E). Hepatocytes cultured in a collagen sandwich configuration remain in chord-like arrays and show fewer signs of cell spreading compared to rigid and gelled collagen substratum. Note the presence of an elaborate, anastomotic canalicular network which circumscribes most of the cells in the culture (arrow). Bar = 50 μ m.

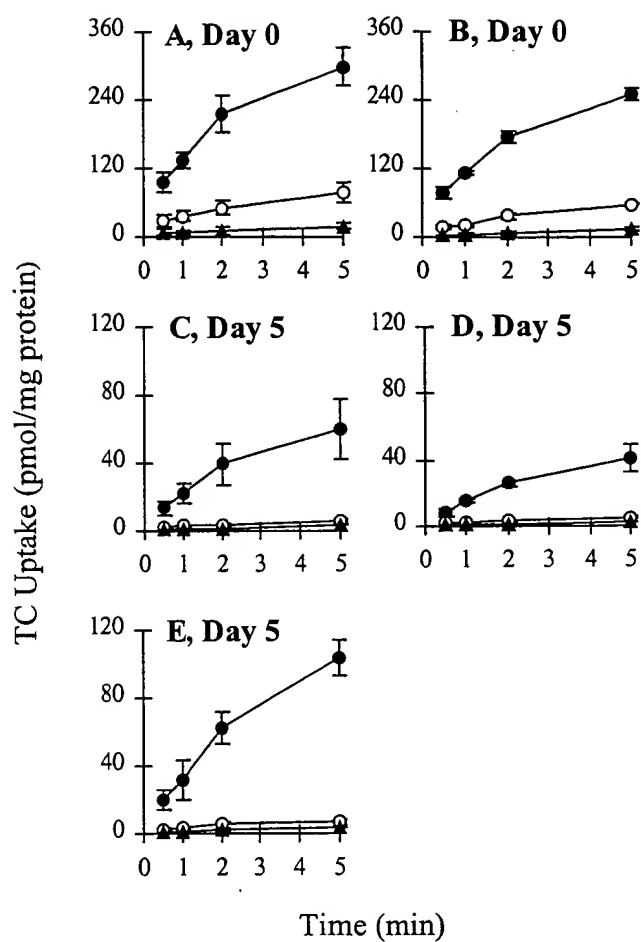


Figure 2-3. Temperature- and Na⁺-dependence of [³H]taurocholate (1 μM) uptake in cultured hepatocytes maintained under different matrix conditions as described in Figure 2 for either 2-4 hr (A, B) or 5 days (C, D & E) on a rigid collagen substratum (B, D), a gelled collagen substratum without an overlay of extracellular matrix (A, C), or between two layers of gelled collagen (E). Symbol ●, ○, and ▲ represents uptake in HBSS at 37°C, in Na⁺-free HBSS at 37°C, and in HBSS at 4°C, respectively.

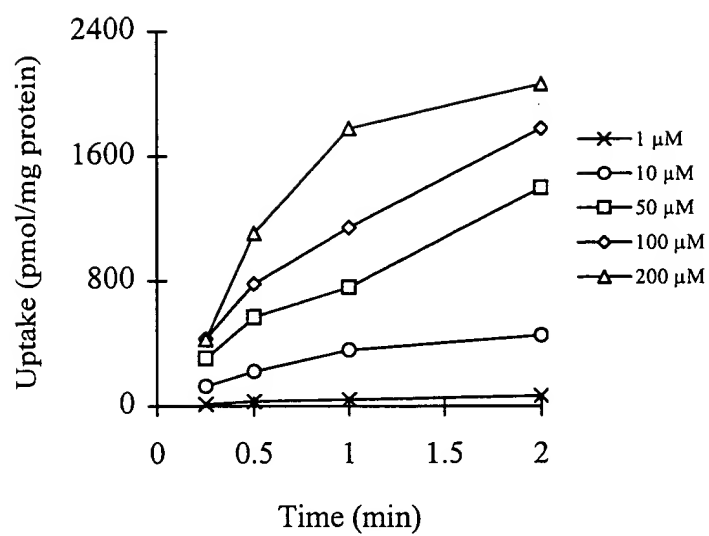


Figure 2-4. Concentration-dependent cumulative $[^3\text{H}]$ taurocholate uptake vs. time in day-5 hepatocyte cultures maintained in a sandwich configuration (n=2, range < 15% of average).

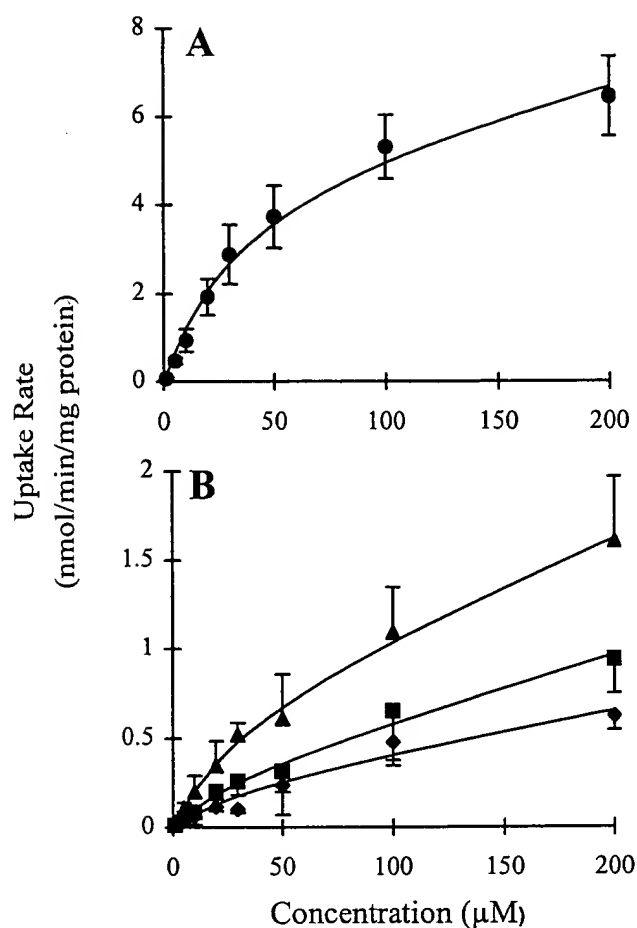


Figure 2-5. Concentration-dependent initial rate of $[^3\text{H}]$ taurocholate uptake (in the presence of sodium) in day-0 hepatocytes cultured on a gelled collagen substratum (A, ●), in day-5 hepatocytes cultured in a sandwich configuration (B, ▲), day-5 hepatocytes cultured on a gelled collagen substratum (B, ■) and day-5 hepatocytes cultured on a rigid collagen substratum (B, ◆). Curved lines represent the fit of a combined Michaelis-Menten and first-order function to the data.

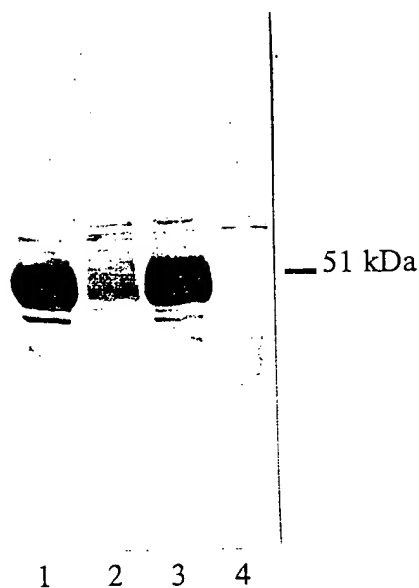


Figure 2-6. Immunoblot analysis of Ntcp in primary cultured rat hepatocytes. All lanes were loaded with 50 μ g crude membrane protein isolated from cultured hepatocytes. Lanes 1 and 3 were from hepatocytes cultured for 2-4 hr on a gelled collagen substratum and on a rigid collagen substratum, respectively. Lanes 2 and 4 were from day-5 hepatocytes cultured in a sandwich configuration and on a rigid collagen substratum, respectively. Ntcp, a single broad band at 51 kDa was detected in hepatocytes cultured for 2-4 hr or hepatocytes cultured in a sandwich configuration for 5 days, but was not detected in hepatocytes cultured on a rigid collagen substratum for 5 days.

CHAPTER 3

BILIARY EXCRETION IN PRIMARY RAT HEPATOCYTES CULTURED IN A COLLAGEN-SANDWICH CONFIGURATION

This chapter has been submitted to the *American Journal of Physiology*, and is presented in the style of that journal.

ABSTRACT

The objective of the present investigation was to examine the functional re-establishment of polarity in freshly isolated hepatocytes cultured between 2 layers of gelled collagen in a sandwich configuration. Immunoblot analysis demonstrated that a significant amount of canalicular multispecific organic anion transport protein was maintained in day-5 hepatocytes cultured in a sandwich configuration. Fluorescein-labeled taurocholate and carboxydichlorofluorescein were excreted into and concentrated in the bile canalicular lumen of day-5 sandwich-cultured hepatocytes resulting in formation of fluorescent networks in standard buffer (intact bile canaliculi). Confocal microscopy studies demonstrated that: (1) carboxydichlorofluorescein that had concentrated in the canalicular lumen was released into the incubation buffer in the presence of Ca^{2+} -free buffer (disrupted bile canaliculi), (2) rhodamine-dextran, an extracellular space marker, was only able to diffuse into the canalicular lumen in the presence of Ca^{2+} -free buffer (disrupted bile canaliculi). The cumulative uptake of [^3H]taurocholate in day-5 sandwich-cultured hepatocytes was significantly higher in standard buffer compared to Ca^{2+} -free buffer, due to accumulation of taurocholate in canalicular spaces. When [^3H]taurocholate was preloaded in the day-5 sandwich-cultured hepatocytes, taurocholate efflux was greater in Ca^{2+} -free compared to standard buffer. The Biliary Excretion Index of taurocholate, equivalent to the percentage of retained taurocholate in the canalicular networks increased from ~8% in cultured hepatocytes at day-0 to ~60% in cultured hepatocytes at day-5. In summary, hepatocytes cultured in a sandwich configuration for 4-5 days re-establish functional polarity and represent a novel *in vitro* model to study biliary excretion.

INTRODUCTION

Biliary excretion of substrates is a complex process *in vivo* involving translocation across sinusoidal membranes, movement through the cytoplasm, and transport across the canalicular membrane. For many cholephilic compounds, transport across the canalicular membrane is the rate-limiting step in overall excretion (11). Many endogenous and exogenous compounds undergo hepatic uptake and biliary excretion via carrier-mediated transporters. A variety of techniques, involving the intact liver *in vivo*, the isolated perfused liver, isolated hepatocyte suspensions, short-term cultured hepatocyte couplets, membrane vesicles, and isolated transport proteins, have been employed to study these transport processes (25). Freshly isolated hepatocytes, utilized to investigate transport mechanisms, represent a very versatile technique for studying the effects of driving forces, protein binding, and competition by other drugs for transport processes (4). In addition, freshly isolated hepatocytes have been utilized to examine the processes of biliary excretion. By measuring the efflux of bile acids in hepatocyte suspensions, Tarao *et al.* (30) demonstrated that cholestasis impairs biliary excretion of bile acids. Similarly, Oude Elferink *et al.* (24) utilized hepatocyte suspensions to study ATP-dependent efflux of oxidized glutathione and dinitrophenyl-glutathione. Studenberg and Brouwer (28) demonstrated the effects of *p*-hydroxyphenobarbital glucuronide on the canalicular excretion of acetaminophen glucuronide in hepatocyte suspensions. Nevertheless, the interpretation of these experiments is hampered by the fact that substrate efflux from isolated hepatocytes may be mediated by sinusoidal as well as canalicular excretion mechanisms. Because of the difficulties in differentiating between sinusoidal efflux and canalicular excretion in isolated hepatocytes,

most transport studies utilizing hepatocyte suspensions are limited to investigating hepatic uptake processes (26).

Short-term (3-8 hr) cultured hepatocyte couplets have been employed to examine the biliary excretion of fluorescent compounds utilizing fluorescence microscopy. Graf and Boyer demonstrated that polarized or vectorial transport function was restored and the canalicular lumen was sealed in hepatocyte couplets (10). However, the application of these techniques is limited because the substrate must contain a fluorescent chromophore. Historically, long-term cultures of hepatocytes (more than 24 hr) have not been a suitable model for studying hepatobiliary transport due to the rapid deterioration of transport properties and other liver specific functions, and failure to maintain normal hepatocyte morphology (11, 15). For instance, Na^+ -dependent taurocholate uptake deteriorated within 3 days to 4% of the uptake exhibited by hepatocytes cultured for 3 hr (21). Likewise, hepatocyte-derived cell lines often lack liver-specific transport functions (25). The functional activity of canalicular transporters has not been examined in long-term cultured hepatocytes under conventional conditions due to deterioration in transport function and the lack of a substantial canalicular domain.

Many studies have been conducted to examine the influence of culture conditions on the expression of the liver-specific phenotype in hepatocyte cultures, including changes in medium composition, co-culture with other epithelial cells, addition of chemical modulators, and alteration of the extracellular matrix environment (17). Primary rat hepatocytes cultured between two layers of gelled collagen form extensive bile canalicular networks and represent a successful approach to maintaining liver specific functions including albumin secretion,

cytochrome P-450 enzyme induction and bile acid uptake (7, 15, 16, Chapter 2). Recently, it was established that hepatocytes cultured in a collagen-sandwich configuration for 5 days exhibited functional bile acid transport and partially maintained Na^+ /taurocholate cotransporting polypeptide (Ntcp) (Chapter 2). In contrast, hepatocytes cultured under conventional conditions were unable to maintain Ntcp. More importantly, the hepatocytes cultured in a collagen-sandwich configuration formed extensive bile canalicular networks (15, Chapter 2). Talamini *et al.* demonstrated that hepatocytes cultured in a sandwich configuration maintain functional polarity and form a sealed canalicular lumen (29). This model system allows for differentiation between sinusoidal and canalicular transport processes and, thus, may represent a novel tool for investigating the hepatobiliary disposition of substrates.

Several ATP-dependent primary active transport systems on the canalicular membrane have been characterized or postulated (25). The ATP-dependent canalicular multispecific organic anion transporter (cMOAT, canalicular multidrug resistance protein or Mrp 2) preferentially transports di- and multivalent organic anions other than bile acids, including glucuronide and glutathione conjugates (2, 13, 24). An ATP-dependent canalicular bile acid transporter that transports taurocholate in isolated rat canalicular liver plasma membrane vesicles has been described (1, 25). Sister of P-glycoprotein may represent the ATP-dependent taurocholate carrier on the canalicular membrane (8). In the present study, the maintenance of cMOAT in sandwich-cultured hepatocytes was examined with immunoblot analysis. The functional activity of cMOAT and the canalicular bile acid transporter were examined in hepatocytes cultured in a collagen-sandwich configuration with

the model substrates carboxydichlorofluorescein, as well as taurocholate and fluorescein-labeled taurocholate, respectively. The time course of re-establishment of vectorial transport was assessed quantitatively. Moreover, the utility of this *in vitro* model system to quantitate biliary excretion of substrates was examined.

MATERIALS AND METHODS

Chemicals. Taurocholate and dexamethasone were purchased from Sigma Chemical Co. (St. Louis, MO). [^3H]Taurocholate (3.4 Ci/mmol) and [^{14}C]salicylic acid (salicylate) (120 mCi/mmol) were obtained from Dupont New England Nuclear (Boston, MA). Carboxydichlorofluorescein and rhodamine-dextran (MW 10 kDa) were obtained from Molecular Probes, Inc. (Eugene, OR). Fluorescein-labeled taurocholate was prepared by Dr. R. L. Bugianesi and kindly provided by Dr. C. P. Sparrow (Merck & Co., Inc., Rahway, NJ). Collagenase (type I, class I) was obtained from Worthington Biochemical Corp. (Freehold, NJ). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum and insulin were purchased from Gibco (Grand Island, NY). Rat tail collagen (type I) was obtained from Collaborative Biomedical Research (Bedford, MA). Sodium dodecyl sulfate (SDS)-polyacrylamide gel and nitrocellulose transfer membrane (0.45 μm) were purchased from Bio-Rad Laboratories (Hercules, CA). An anti-serum against the C-terminus of cMOAT was raised by immunizing rabbits with the peptide AGIENVNHTL, which was coupled at the C-terminus with an additional C to keyhole limpet hemocyanin with MBS (Neosystem Laboratories, Strasbourg, France). Enhanced chemiluminescence (ECL) detection kit and Hyperfilm-ECL were purchased from Amersham Life Sciences (Buckinghamshire, England). All other chemicals and reagents were of analytical grade and were readily available from commercial sources.

Animals. Male Wistar rats (250-280 g) from Charles River (Raleigh, NC) were used as liver donors. They were allowed free access to food (Laboratory rodent diet #5001, PMI Feeds, Inc., St. Louis, MO) and water, and were housed in a constant alternating 12-hr light

(6:00 AM to 6:00 PM) and dark cycle. All procedures were approved by the Institutional Animal Care and Use Committee. ____

Preparation of Culture Dishes. Plastic culture dishes (60 mm) were precoated with rat tail collagen at least 1 day prior to preparing the hepatocyte cultures. To obtain a gelled collagen substratum, ice-cold neutralized collagen solution (0.1 ml, 1.5 mg/ml, pH 7.4) was spread onto each culture dish. Freshly coated dishes were placed at 37°C in a humidified incubator for approximately 1 hr to allow the matrix material to gel, followed by addition of 3 ml DMEM to each dish and storage in a humidified incubator.

Isolation and Culture of Rat Hepatocytes. Hepatocytes were isolated with a two-step perfusion method as reported previously (Chapter 2). Rats were anesthetized with ketamine and xylazine (60 and 12 mg/kg i.p., respectively) prior to portal vein cannulation. The liver was perfused *in situ* with oxygenated calcium-free Krebs-Henseleit bicarbonate buffer containing 5.5 mM glucose for 10 min at 37°C followed by perfusion with Krebs-Henseleit bicarbonate buffer containing collagenase type I (0.5 mg/ml) for 10 min. Hepatocytes were released by shaking the liver gently in 100 ml DMEM. The released cells were filtered through a sterile nylon 70- μ m filter into two 50-ml centrifuge tubes. The hepatocyte suspensions were centrifuged at 50 \times g for 3 min. The cell pellet was resuspended in 25 ml DMEM and an equal volume of 90% isotonic Percoll (pH 7.4); the resulting cell suspension was centrifuged at 150 \times g for 5 min. The pellet was resuspended in 50 ml DMEM and cell suspensions were combined into one tube followed by centrifugation at 50 \times g for 3 min. Cell suspensions were prepared with DMEM containing 5% fetal calf serum, 1 μ M dexamethasone and 4 mg/L insulin. Hepatocyte viability was determined by trypan blue

exclusion. Only those hepatocyte preparations with viability greater than 90% were utilized for preparation of cultures. Hepatocyte suspensions were added to the pre-coated dishes at a density of 2×10^6 cells/60-mm dish. Approximately 1 hr after plating the cells, the medium was aspirated and 3 ml fresh DMEM was added. For hepatic transport studies, hepatocytes that had been seeded for 3-5 hr without collagen overlay were defined as day-0 or short-term cultured hepatocytes.

To prepare sandwich-cultured hepatocytes, neutralized collagen solution (0.1 ml, 1.5 mg/ml, pH 7.4) was added to the monolayers 24 hr after the cells were seeded. Cultures with collagen overlay were incubated for 45 min at 37°C in a humidified incubator to allow the collagen to gel before addition of DMEM. Medium was changed on a daily basis until the fifth day after the cells were seeded. These hepatocytes were referred to as day-5 or long-term cultured hepatocytes.

Immunoblot Analysis. To prepare crude plasma membrane, hepatocyte cultures were rinsed once with 3-ml ice-cold Hanks' balanced salt solution (standard buffer). Cells were collected by scraping into hypotonic lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 0.4 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, and 10 µg/ml leupeptin) and incubated in an ice bath for 15 min. The swollen cells were disrupted with 30 strokes in a tightly fitting Dounce homogenizer. The nuclei were removed by centrifugation at 400×g for 10 min at 4°C. The pellet obtained by subsequent centrifugation at 30,000×g for 30 min at 4°C was used as the crude membrane fractions (9). Rat canalicular liver plasma membrane

fractions were isolated by standard methods described by Meier and Boyer (23) and used as a standard for the immunoblot analysis.

Proteins from the crude membrane fractions (50 μ g) were subjected to 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis by the method of Laemmli (19). After proteins were transferred electrophoretically from SDS gels to nitrocellulose membranes, the blots were blocked with Tris-buffered saline containing 0.05% Tween 20 and 5% non-fat dry milk for 1 hr at room temperature. Rabbit anti-serum raised against the C-terminus of cMOAT was used for the primary antibody in the immunoblot analysis. The specificity of this anti-serum has been confirmed previously in vesicles derived from Sf9 cells expressing cMOAT and isolated canalicular plasma membrane vesicles (data not shown). The blots were probed with the polyclonal anti-cMOAT rabbit serum at 1:4000 dilution. Antibody binding was visualized with horseradish peroxidase-conjugated donkey anti-rabbit Immunoglobulin G serum at 1:2000 dilution, followed by detection with Amersham enhanced chemiluminescence kit and exposure on Amersham Hyperfilm according to the manufacturer's instructions. The following molecular weight standards were used: myosin 205 kDa; β -galactosidase 118 kDa; bovine serum albumin, 85 kDa; and ovalbumin, 47 kDa (Bio-Rad Laboratories, Hercules, CA).

Fluorescence Microscopy. For the carboxydichlorofluorescein biliary excretion studies, hepatocytes were incubated in standard buffer at 37°C for 10 min. Subsequently, each dish received 3-ml standard buffer containing 1 μ g/ml of carboxydichlorofluorescein diacetate. The hepatocytes were incubated at 37°C for 5 min. Fluorescein-labeled taurocholate studies were performed utilizing a similar protocol except that the final

incubation lasted for 10 min. After loading the substrate, each dish was rinsed 4 times with 3 ml standard buffer to remove extracellular substrate prior to viewing with a Leitz fluovert FU fluorescence microscope.

Laser Scanning Confocal Microscopy. Fluorescence images were collected with a Bio-Rad (Cambridge, MA) MRC-600 laser scanning confocal microscope equipped with an argon/krypton multiline laser and mounted on a Nikon (Garden City, NY) diaphot inverted microscope. The objective lens was a 60× NA 1.4 planapochromat. A pinhole setting of 3 to 4 was used to maximize optical sectioning, producing confocal optical sections of 3 μm in thickness. Confocal machine settings (gain, black level and neutral density filters) were set to maximize the dynamic range between background and the more intense canalicular fluorescence. Green fluorescence of fluorescein excited at 488 nm was collected through a 515-nm long-pass barrier filter (fluorescein channel). Red fluorescence of rhodamine-dextran excited at 568 nm was collected through a 585-nm long-pass barrier filter (rhodamine channel) (18). Sandwich-cultured hepatocytes (day-5) were rinsed twice with 3-ml standard buffer and preloaded with carboxydichlorofluorescein by addition of 10 μg of carboxydichlorofluorescein diacetate in 3-ml standard buffer and incubated at 37°C for 10 min. Thereafter, the monolayers were rinsed twice with 3-ml standard buffer to remove extracellular substrate. Rhodamine-dextran (2 mg/ml in standard buffer) was added to the day-5 sandwich-cultured hepatocytes. Images were collected immediately in the fluorescein channel and the rhodamine channel, respectively. Subsequently, the cultures were rinsed with 2 ml of Ca^{2+} , Mg^{2+} -free Hanks' balanced salt solution containing 1 mM EGTA (Ca^{2+} -free buffer) twice, and the monolayers were maintained for 10 min in 2 mg/ml rhodamine-

dextran solution prepared in Ca^{2+} -free buffer prior to again collecting the images in the fluorescein and rhodamine channels, respectively.

Efflux Studies in Sandwich-Cultured Hepatocytes. Hepatocytes cultured in a collagen-sandwich configuration were incubated in 3 ml standard buffer at 37°C for 10 min. Each dish received 3 ml standard buffer containing 1 μM [^3H]taurocholate or 3.6 μM [^{14}C]salicylate followed by incubation at 37°C for 10 min. Subsequently, the incubation buffer was removed and cultures were washed 4 times with 3 ml ice-cold standard buffer to quench the transport processes and remove extracellular substrate. Efflux was initiated by addition of 3 ml standard buffer or Ca^{2+} -free buffer to each dish. Aliquots of efflux buffer (0.1 ml) were removed at designated times and analyzed by liquid scintillation spectrometry.

Uptake Studies in Sandwich-Cultured Hepatocytes. Hepatocytes cultured in a collagen-sandwich configuration were incubated in 3 ml standard buffer or Ca^{2+} -free buffer at 37°C for 10 min. After removing the incubation buffer, uptake was initiated by addition of 3 ml standard buffer containing 1 μM [^3H]taurocholate or 1 μM [^{14}C]salicylate to each dish. After incubation for designated times, cumulative uptake was terminated by aspirating the incubation solution and rinsing 4 times with 3 ml ice-cold standard buffer to remove extracellular substrate. Each rinse lasted 10 seconds. After washing, 2 ml of 1% Triton X-100 solution was added to culture dishes to lyse cells by shaking the dish on a shaker for 20 min at room temperature. An aliquot (1 ml) of lysate was analyzed by liquid scintillation spectrometry. All values for taurocholate uptake into cell monolayers were corrected for nonspecific binding to the collagen by subtracting taurocholate uptake determined in the appropriate control dishes in the absence of cells as described previously (Chapter 2).

Protein Assay. Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA) was used to determine the protein concentration in the culture extracts using bovine serum albumin as standard. Triton X-100 (1%) did not interfere with the assay.

Data analysis. Efflux or uptake data were normalized to the protein content and expressed as mean \pm SD from 3-4 separate preparations of hepatocytes. Differences between experimental groups were analyzed by multivariate analysis of variance. A *P* value of < 0.05 was considered significant.

RESULTS

Effect of an Upper Layer of Collagen Gel on the Morphology of Cultured Hepatocytes. Hepatocytes cultured on a collagen gel substratum for 5 days displayed signs of cell spreading and deterioration of normal cuboidal morphology, and did not form significant interconnecting networks of bile canaliculi (Figure 3-1A). The margins of many hepatocytes viewed with a phase contrast microscope were not clearly defined. In contrast, day-5 sandwich-cultured hepatocytes remained in chord-like arrays, did not display significant signs of cell spreading, and showed anastomosing networks of bile canaliculi that circumscribed most of the cells in the culture (Figure 3-1B).

Immunoblot Analysis of cMOAT in Hepatocyte Membranes. Immunoblot analysis of canalicular plasma membranes isolated from rat liver, and crude membrane fractions prepared from rat hepatocytes cultured on a gelled substratum for 2-4 hr, probed with antibody raised against the C-terminal peptide sequence of rat cMOAT, showed a band with a molecular mass of approximately 190 kDa (Figure 3-2, lane 1 and 2). Immunoblot analysis of crude membrane fractions isolated from hepatocytes cultured in a collagen-sandwich configuration for 5 days also showed a band with molecular mass of approximately 190 kDa (Figure 3-2, lane 3). A slight increase in the molecular weight of cMOAT by 10-15 kDa was observed in the day-5 sandwich-cultured hepatocytes.

Polarized Excretion of Carboxydichlorofluorescein in Sandwich-Cultured Hepatocytes. The vectorial excretory activity of sandwich-cultured hepatocytes was examined with the fluorescent cMOAT substrate, carboxydichlorofluorescein. Immediately after addition of carboxydichlorofluorescein diacetate to hepatocyte monolayers cultured for 4 hr

(day-0) or hepatocytes cultured in a sandwich configuration for 5 days, strong fluorescence was observed in the cell interior. In day-0 hepatocytes, the carboxydichlorofluorescein remained localized predominantly in the cytoplasm of hepatocytes (Figure 3-3A and 3C). However, fluorescence was concentrated between a few hepatocytes, suggesting the existence of residual sealed bile canalicular spaces between some adjacent hepatocytes with functional activity of cMOAT (Figure 3-3A). In contrast, in day-5 sandwich-cultured hepatocytes, the strong intracellular fluorescence translocated rapidly into bile canalicular networks that surrounded each hepatocyte (Figure 3-3B). These results suggest that cMOAT is functionally active in the canalicular membrane of hepatocytes cultured in a sandwich configuration for 5 days, and that the canalicular network represents a separate compartment that is able to concentrate substrates.

Preliminary studies demonstrated that the fluorescence concentrated in the canalicular networks, as described above, disappeared within a few minutes in Ca^{2+} -free buffer, suggesting that the bile canalicular space was sealed by tight junctions. Ca^{2+} modulation appeared to disrupt the tight junctions allowing the fluorescent probe in the canalicular lumen to diffuse into the medium. Confocal microscopy was employed to demonstrate that the fluorescence in the canalicular networks in Figure 3-3B was due to transport of substrate from the cytoplasm into the canalicular lumen, which was separated from the incubation medium by junctional complexes. Carboxydichlorofluorescein, which is actively transported into bile, and rhodamine-dextran, an extracellular space marker, were employed simultaneously in this study to examine canalicular excretion and the integrity of the canalicular lumen in day-5 sandwich-cultured hepatocytes.

After incubation in carboxydichlorofluorescein diacetate solution for 10 min, the monolayer was viewed with a laser scanning confocal microscope. In a process of confocal sectioning from the top to the bottom of the monolayer, an image on each focal plane appeared sequentially. In the fluorescein channel, a dark field was observed first; then fluorescent networks appeared (Figure 3-4A) and were followed by a dark field. In contrast, in the rhodamine channel, a bright field was observed first, then a dark area appeared (which matched the location of hepatocytes and canalicular spaces viewed under the phase contrast microscope) surrounded by a bright domain (Figure 3-4B); subsequently a bright field was observed. These image planes delineated the extracellular space above the monolayer, the intracellular and bile canalicular space across the monolayer, and the extracellular space below the monolayer. These results confirmed that carboxydichlorofluorescein was localized predominantly in the bile canaliculi, while rhodamine-dextran was excluded from the hepatocytes and the bile canalicular space. The fluorescent images were stable for at least 10 min, suggesting that carboxydichlorofluorescein was unable to diffuse readily from the bile canalicular lumen into the extracellular medium, and that rhodamine-dextran was unable to penetrate into the bile canalicular space from the extracellular medium. In contrast, after incubation in Ca^{2+} -free buffer for 5 min, the fluorescent networks in the fluorescein channel were no longer visible (Figure 3-4C) but the fluorescent networks in the rhodamine channel were visible (Figure 3-4D). Disruption of the tight junctions that maintain the integrity of the canalicular space by Ca^{2+} modulation enabled carboxydichlorofluorescein to move into the incubation medium and rhodamine-dextran to move into the canalicular space, respectively, based on favorable concentration gradients.

Polarized Excretion of Taurocholate in Sandwich-Cultured Hepatocytes. The vectorial transport of bile acids was examined with fluorescein-labeled taurocholate (Figure 3-5), a fluorescent bile acid. Preliminary studies demonstrated that the fluorescein-labeled taurocholate was stable under the experimental conditions. Hepatocytes cultured for 5 days in a sandwich configuration were incubated with fluorescein-labeled taurocholate for 10 min. Fluorescence was localized predominantly in the canalicular spaces (Figure 3-6), demonstrating that fluorescein-labeled taurocholate was transported across the canalicular membrane against a concentration gradient. These data indicate that active vectorial bile acid transport was re-established in day-5 sandwich-cultured hepatocytes.

The efflux and cumulative uptake of [^3H]taurocholate in sandwich-cultured hepatocytes with intact and disrupted bile canaliculi was examined to further demonstrate vectorial bile acid transport in day-5 sandwich-cultured hepatocytes. [^{14}C]Salicylate was utilized as a marker for simple diffusion because the biliary excretion of salicylate is negligible *in vivo* (20, 23). After pre-loading hepatocyte monolayers with [^3H]taurocholate or [^{14}C]salicylate, substrate efflux was examined by measuring the amount of substrate appearing in standard or Ca^{2+} -free buffer (Figure 3-7). In hepatocytes cultured for approximately 4 hours, [^3H]taurocholate efflux in standard buffer and Ca^{2+} -free buffer was similar. However, in day-5 sandwich-cultured hepatocytes, [^3H]taurocholate efflux in Ca^{2+} -free buffer was significantly higher than that in standard buffer ($p < 0.01$). In contrast, [^{14}C]salicylate efflux failed to show any significant differences in standard and Ca^{2+} -free buffer ($p > 0.05$).

In cumulative uptake studies, sandwich-cultured hepatocytes were preincubated in standard or Ca^{2+} -free buffer for 10 min before quantitating [^3H]taurocholate—and [^{14}C]salicylate in the cultures (Figure 3-8). At day-0 and day-5, [^3H]taurocholate uptake at various incubation times was greater in standard buffer than in Ca^{2+} -free buffer ($p < 0.05$). At day-0, cumulative taurocholate uptake at 10 min in standard buffer was approximately 10% greater compared to in Ca^{2+} -free buffer. At day-5, the cumulative uptake in standard buffer was 120% greater compared to in Ca^{2+} -free buffer. In contrast, no significant differences were observed in the cumulative uptake of [^{14}C]salicylate by hepatocytes incubated in standard and Ca^{2+} -free buffer.

Time Course of Re-establishment of Polarized Excretory Function in Sandwich-Cultured Hepatocytes. In order to examine the time-course of the re-establishment of polarized excretory function in sandwich-cultured hepatocytes, cumulative uptake of the bile acid [^3H]taurocholate (1 μM) at 10 min in standard and Ca^{2+} -free buffer was assessed at various times after seeding (Figure 3-9A). After the isolated hepatocytes were plated on the gelled collagen substratum, the cumulative uptake of taurocholate in standard buffer was greater than Ca^{2+} -free buffer, and was maintained at similar levels for the first 5 hr. Cumulative taurocholate uptake in both standard buffer and Ca^{2+} -free buffer declined over time in culture. By 48 hr, the cumulative taurocholate uptake in standard buffer was approximately 60% of that at 5 hr. Between 96 and 120 hr, taurocholate retention was approximately 25% of that at 5 hr.

Use of this model system to assess biliary excretory function must account for the deterioration in substrate uptake over time in culture. In sandwich-cultured hepatocytes, the

cumulative uptake of substrate in standard buffer represents the amount of substrate localized in the cytoplasm and bile canalicular networks; the cumulative uptake of substrate in Ca^{2+} -free buffer represents the amount of substrate localized intracellularly. Polarized excretion of substrates may be quantitatively assessed based on the following formula:

$$\text{Biliary Excretion Index} = \frac{\text{Uptake}_{\text{standard buffer}} - \text{Uptake}_{\text{Ca}^{2+}\text{-free buffer}}}{\text{Uptake}_{\text{standard buffer}}} \times 100\%$$

where, $\text{Uptake}_{\text{standard buffer}}$ represents the cumulative uptake of substrate at 10 min in standard buffer, and $\text{Uptake}_{\text{Ca}^{2+}\text{-free buffer}}$ represents the cumulative uptake of substrate at 10 min in Ca^{2+} -free buffer.

The Biliary Excretion Index of taurocholate in sandwich-cultured hepatocytes at various culture times was calculated (Fig 9B). In short-term cultured hepatocytes, the Biliary Excretion Index of taurocholate was approximately 8%. The Biliary Excretion Index of taurocholate increased with culture time to approximately 60% at 72 hr. Thereafter, the Biliary Excretion Index of taurocholate appeared to reach a plateau.

DISCUSSION

Hepatocytes cultured in a conventional configuration (on rigid collagen) dedifferentiate and rapidly lose hepatic transport activity and other liver specific functions. However, hepatocytes cultured in a collagen sandwich configuration retain some liver specific functions such as albumin secretion and cytochrome P-450 enzyme induction, and form extensive bile canalicular networks (7, 15). The bile canaliculi re-established in culture have morphological and biochemical characteristics similar to those observed *in vivo*, including microvilli in the bile canaliculi and localization of specific enzymes in the apical membrane (*e.g.*, Mg^{2+} -ATPase, aminopeptidase and alkaline phosphatase) (15, 29).

In the present study, the effect of an upper layer of collagen gel on the morphology of cultured hepatocytes was examined. Even though the substratum was identical, extensive bile canalicular networks only were established in the hepatocytes cultured with a top layer of gelled collagen (sandwich configuration); canalicular networks were not formed in the hepatocytes plated on a collagen gel without the collagen overlay.

Hepatocytes cultured in a sandwich configuration may serve as a novel *in vitro* model to study hepatobiliary transport processes. Cultured hepatocytes have numerous advantages over other model systems, including high efficiency and easy manipulation of experimental conditions for assessing hepatobiliary transport. In addition, the regulation of transport systems under different culture conditions can be examined in long-term cultured hepatocytes. The present investigation represents the first attempt to examine the functional activity of canalicular transporters in long-term cultured hepatocytes.

The canalicular isoform of the multidrug resistance protein (cMOAT) from rat liver has been cloned and sequenced; cMOAT contains 1541 amino acids with a calculated molecular mass of 173,318 Da and 11 predicted N-glycosylation sites (3). The apparent molecular mass of cMOAT in rat liver is approximately 190 kDa due to glycosylation (3, 31). In the present work, the molecular mass of cMOAT in day-0 and day-5 cultured hepatocytes was approximately 190 kDa. The slightly higher molecular mass in day-5 sandwich-cultured hepatocytes may be due to altered post-translational processing of this protein during culture. Trauner *et al.* (31) noted that the molecular mass of cMOAT increased 10-15 kDa in common bile duct ligated rats and suggested that post-translational processing of this protein may be altered during cholestasis.

Recent studies have been demonstrated that the amount of Ntcp was significantly lower in day-5 sandwich-cultured hepatocytes compared to day-0 cultured hepatocytes (Chapter 2). Unlike Ntcp, cMOAT did not deteriorate significantly in long-term cultured hepatocytes, suggesting that the expression of cMOAT and Ntcp follow different regulation mechanisms. Liang *et al.* (21) demonstrated that decreased Na⁺-dependent taurocholate uptake in cultured hepatocytes was parallel to the reduction in mRNA of the transport protein. Whether the maintenance of functional activity of cMOAT in hepatocytes cultured in a sandwich configuration is due to effects on transcription or post-transcription of the transport protein is the subject of ongoing investigations.

Immunoblot analysis demonstrated the existence of cMOAT protein in hepatocytes cultured in a sandwich configuration for 5 days. Subsequent studies were conducted to examine vectorial transport of cMOAT substrates in the sandwich-cultured hepatocyte

monolayers. Kitamura *et al.* (14) demonstrated that carboxydichlorofluorescein is a cMOAT substrate; biliary excretion of this compound is negligible in TR⁻ rats (cMOAT-deficient mutant Wistar rats). In the present study, carboxydichlorofluorescein was employed as a model substrate to examine the functional activity of cMOAT. Carboxydichlorofluorescein diacetate, which exhibits only weak fluorescence, was utilized due to its rapid penetration into the hepatocyte plasma membrane. Carboxydichlorofluorescein diacetate is hydrolyzed readily in the cytoplasm by intracellular esterases to a highly fluorescent product, carboxydichlorofluorescein (12).

The fluorescence of carboxydichlorofluorescein is sensitive to pH (12). Any analysis based on the intensity of carboxydichlorofluorescein fluorescence should consider the effects of pH. However, less than a 0.3 pH unit difference has been found between cytosol and bile canaliculi in hepatocyte couplets (27). Although carboxydichlorofluorescein has been used for pH determinations in acidic organelles, its fluorescence intensity is not altered markedly between pH 7.1-7.4. The fluorescence of carboxydichlorofluorescein at pH 7.4 is only 10%-20% higher than at pH 7.1 at maximum emission wavelength (12). Inasmuch as the fluorescence of carboxydichlorofluorescein is used as a qualitative probe to localize carboxydichlorofluorescein cellular distribution, the slight pH gradient between the cytosol and the canaliculi would not affect the conclusions of this study.

In short-term cultured hepatocytes, significant fluorescence was retained intracellularly; in contrast, in day-5, sandwich-cultured hepatocytes, negligible fluorescence was observed in the cytoplasm (Figure 3-3A and B, respectively). These results suggest that the functional activity of cMOAT is lower in short-term cultured hepatocytes compared to

long-term cultured hepatocytes, probably due to endocytosis of the membrane protein after isolation and a decrease in cMOAT on the plasma membrane (10). In short-term cultured hepatocytes, fluorescence is concentrated between some of the hepatocytes, suggesting that the residual bile canaliculi have sealed and the hepatocytes have maintained some functional activity of cMOAT. These observations are in agreement with studies in hepatocyte couplets demonstrating that sealed canalicular spaces form approximately 3-5 hr after seeding (10). The fluorescent carboxydichlorofluorescein did not accumulate in the spaces between the majority of short-term cultured hepatocytes, suggesting that the residual bile canaliculi may not be sealed completely, or that cMOAT is not functionally active in most of the short-term cultured hepatocytes. In day-5, sandwich-cultured hepatocytes, the fluorescent marker accumulated throughout the canalicular spaces, demonstrating the functional activity of cMOAT on the canalicular membrane and functional integrity of the bile canalicular networks. In contrast, hepatocytes cultured under conventional culture conditions on a rigid gelled collagen for 5 days failed to show these fluorescent networks (data not shown). However, some sporadic fluorescent fragments were observed, suggesting that the canalicular spaces were not completely sealed and/or the functional activity of cMOAT was significantly lower in hepatocytes cultured under conventional conditions compared to hepatocytes cultured in a sandwich configuration.

In short-term cultured hepatocyte couplets, the canalicular lumen is sealed by a tight junction complex (10). LeCluyse *et al.* (15) examined the hepatocyte junctional complex via electron microscopy in hepatocytes cultured in a sandwich configuration for 6 days and demonstrated that the canalicular space is sealed by tight junction-like structures. Recently,

Talamini *et al.* (30) demonstrated the existence of junctional protein, uvomorulin (E-cadherin), in the hepatocytes cultured in a sandwich configuration. In order to determine whether the functional integrity of the bile canalicular networks in hepatocytes cultured in a sandwich configuration is maintained by tight junctional complexes, the effects of extracellular calcium concentration on the stability of the fluorescent networks was examined in the present study with two fluorescent markers, carboxydichlorofluorescein and rhodamine-dextran.

The fluorescence intensity of the networks due to carboxydichlorofluorescein decreased markedly in the first minute after exposure of the hepatocytes to Ca^{2+} -free buffer; fluorescence was not visible at the end of 5 minutes (Figure 3-5C), indicating that the barrier function of the tight junctions had been disrupted within a few minutes. In Ca^{2+} -free buffer, substrate in the canalicular lumen diffused rapidly into the incubation buffer. Moreover, rhodamine-dextran, an extracellular marker, was unable to penetrate into the bile canaliculi in standard buffer. However, in Ca^{2+} -free buffer, rhodamine-dextran readily diffused into the canalicular lumen. These studies indicated that in sandwich-cultured hepatocytes, the tight junctions are an impermeable barrier between the canalicular lumen and the incubation buffer. This barrier can be disrupted within a few minutes by depletion of Ca^{2+} in the incubation medium.

Rapid disruption of the barrier function of tight junctions has been reported by Citi (6) in cultured Madin-Darby canine kidney cells; removal of calcium by exposure to Ca^{2+} -free buffer with 1 mM EGTA reduced the transepithelial electrical resistance within 5 min. In the present study, the disappearance of the fluorescent marker carboxydichlorofluorescein

from the bile canalicular networks after exposure to low concentrations of extracellular calcium appears to be an alternative approach to evaluate the integrity of the tight junctions, analogous to transepithelial electrical resistance measurements utilized in other monolayer systems for evaluating the functional integrity of tight junctions.

Fluorescent microscopy studies with a fluorescent bile acid, fluorescein-labeled taurocholate, demonstrated that this bile acid was taken up by hepatocytes and excreted extensively into the canaliculi of hepatocytes cultured in a sandwich configuration for 6 days. This observation indicated that the polarized transport systems for bile acids were reestablished in the long-term cultured hepatocytes. Furthermore, two experimental approaches were developed to evaluate the biliary excretion of nonfluorescent substrates in the cultured hepatocytes. These two methods are based on the observations that in standard buffer, the integrity of the bile canalicular networks remains intact; in Ca^{2+} -free buffer, the integrity of the canalicular space is disrupted, causing leakage of the canalicular contents.

In the efflux method, substrate was first loaded into hepatocytes followed by measurement of substrate efflux in standard buffer or in Ca^{2+} -free buffer. Theoretically, when a cholephilic compound is taken up into sandwich-cultured hepatocytes, it should be excreted into the canalicular space if the transporter facilitating biliary excretion of this substrate remains functionally active. The efflux rate of a cholephilic compound should be higher in Ca^{2+} -free buffer compared to standard buffer due to disruption of the tight junctions and leakage of substrate from the canalicular space where it has accumulated during substrate loading. In contrast, the efflux rate of a noncholephilic compound should be similar regardless of the extracellular calcium concentrations in the efflux buffer because negligible

amounts of substrate have accumulated in the canalicular space. In the present investigation, the canalicular transport function of bile acids was examined with a cholephilic compound, taurocholate. A noncholephilic compound, salicylate, was utilized as a negative control. At each time point in day-5 sandwich-cultured hepatocytes, the efflux of [^3H]taurocholate was greater in the Ca^{2+} -free buffer compared to standard buffer. In contrast, efflux of [^{14}C]salicylate was not significantly different in Ca^{2+} -free and standard buffer in both day-0 and day-5 cultured hepatocytes. As expected, [^3H]taurocholate and [^{14}C]salicylate efflux in short-term cultured hepatocytes in Ca^{2+} -free and standard buffer was not significantly different because extensive sealed canalicular networks had not formed.

The second experimental approach measured cumulative uptake of substrate. Cultured hepatocytes were preincubated in standard buffer or Ca^{2+} -free buffer for 10 min. Subsequently, cultures were incubated in standard buffer with substrate for designated times, uptake was terminated, and cumulative substrate in the cultures was quantitated. During uptake studies, substrate excreted into the intact bile canalicular networks will be stored there, whereas substrate excreted across the canalicular membrane when the integrity of the canalicular spaces has been disrupted will diffuse back into the incubation medium. Therefore, if cumulative uptake of a cholephilic substrate is greater in cultures preincubated in standard buffer (intact canaliculi) compared to cultures preincubated in Ca^{2+} -free buffer (disrupted canaliculi), then the functional activity of relevant canalicular transporter(s) is maintained. For a noncholephilic substrate, the cumulative uptake of substrate should be identical between the two treatments because biliary excretion of the substrate is negligible.

The cumulative uptake of [^3H]taurocholate was significantly higher in standard buffer relative to Ca^{2+} -free buffer in day-5 sandwich-cultured hepatocytes, suggesting the functional activity of canalicular bile acid transport and integrity of the junctional complexes. As a negative control, [^{14}C]salicylate cumulative uptake in Ca^{2+} -free and standard buffer did not show significant differences in either short- or long-term cultured hepatocytes. Interestingly, the cumulative uptake of [^3H]taurocholate was approximately 10% greater in standard buffer compared to Ca^{2+} -free buffer in short-term cultured hepatocytes. This may be due to the existence of hepatocyte couplets containing sealed canalicular lumens (10).

The differences in substrate efflux or cumulative uptake in standard and Ca^{2+} -free buffer represent the extent of biliary excretion of substrate, assuming that calcium depletion does not alter the hepatobiliary transport of the substrate other than by disruption of the junctional complexes. This assumption appears to be valid because the uptake of bile acids, such as cholate, has been reported to be independent of extracellular calcium concentrations in freshly prepared hepatocyte suspensions (26). Similar results were obtained with taurocholate (data not shown). Furthermore, [^{14}C]salicylate was employed as a simple diffusion marker because it is metabolized extensively by the liver and eliminated as metabolites exclusively in urine (20, 23). Salicylate failed to show any significant difference in all of the treatments, suggesting that modulation of Ca^{2+} concentrations does not alter the diffusional properties of the plasma membrane. Thus, the difference in taurocholate efflux or cumulative uptake in standard and Ca^{2+} -free buffer can be explained by vectorial transport of taurocholate into the canalicular lumen and functional integrity of the canalicular junctional complexes.

One disadvantage of the efflux method is that during efflux studies with Ca^{2+} -free buffer, substrate in the canalicular space is released into the medium and can be taken up again across the basolateral membrane. The difference of efflux will underestimate the actual amount of substrate in the canalicular networks. In contrast, when measuring cumulative uptake, the substrate localized in the canalicular space releases back into the medium in the presence of Ca^{2+} -free buffer, but substrate concentrations in the medium remain relatively constant. Thus, measurement of cumulative uptake of substrate is more accurate than the efflux method to quantitate the amount of substrate in the canalicular networks.

Accurate assessment of the biliary excretory function of substrates must consider the fact that substrate uptake declines over time in sandwich-cultured hepatocytes. Polarized excretory function may be quantitatively assessed utilizing the Biliary Excretion Index, which represents the percentage of retained substrate in the monolayer that is localized in bile canaliculi. This is similar to the approach utilized by Boyer and Soroka to assess the relative amount of fluorescent bile acid in the bile lumen in short-term cultured hepatocyte couplets (5). In the calculation of the Biliary Excretion Index, biliary excretion of substrate is normalized to the total amount of substrate accumulated in sandwich-cultured hepatocytes. This normalization accounts for variations in uptake activity, and thus, should be an appropriate method to evaluate the vectorial transport of hepatocytes. Theoretically, a large Biliary Excretion Index indicates extensive polarized excretion of substrate. The Biliary Excretion Index increased with time in culture up to 4 to 5 days, suggesting that the functional polarity of sandwich-cultured hepatocytes was gradually established.

Utilizing confocal microscopy and image analysis Boyer and Soroka (5) demonstrated that approximately 6% of cholylglycylamido fluorescein, a fluorescent bile acid, was secreted into the bile canalicular lumen of short-term cultured hepatocyte couplets. The Biliary Excretion Index of taurocholate measured in short-term cultured hepatocytes in our studies was approximately 8%. These data may be due to the existence of hepatocyte couplets with polarized excretion function. The Biliary Excretion Index of taurocholate in long-term cultured hepatocytes was more than six-fold greater than in short-term cultured hepatocytes, demonstrating the re-establishment of polarized excretory function in day-5 sandwich-cultured hepatocyte monolayers. These results are consistent with the previous observation that cultured hepatocytes develop morphological and functional polarity in 4 to 5 days (29).

In addition to utilizing the Biliary Excretion Index to evaluate the functional polarity of cultured hepatocytes, measurement of the Biliary Excretion Index for a given substrate may provide a novel approach to predict the hepatobiliary disposition of that substrate *in vivo*. For example, if the Biliary Excretion Index of a substrate is as high as taurocholate, this substrate may be excreted extensively into bile *in vivo*. However, if the Biliary Excretion Index of a substrate is much less than taurocholate, this substrate may not be secreted as extensively into bile *in vivo*, or the hepatobiliary transport system(s) for this substrate may not be maintained in the sandwich-cultured hepatocytes. Furthermore, the Biliary Excretion Index also may be useful in predicting possible substrate-substrate interactions relevant to hepatobiliary disposition.

In summary, primary cultures of rat hepatocytes maintained in a collagen-sandwich configuration for up to 5 days establish intact canalicular networks, maintain cMOAT, re-

establish polarized excretory function, and appear to express a more normal phenotype compared to conventional cultures. The taurocholate Biliary Excretion Index, a measure of the relative amount of substrate in the bile lumen, may be a useful indicator of polarized excretory function and the relative extent of biliary excretion of a substrate. Therefore, primary cultures of rat hepatocytes maintained in a collagen-sandwich configuration represent a novel *in vitro* tool to study hepatobiliary transport of xenobiotics.

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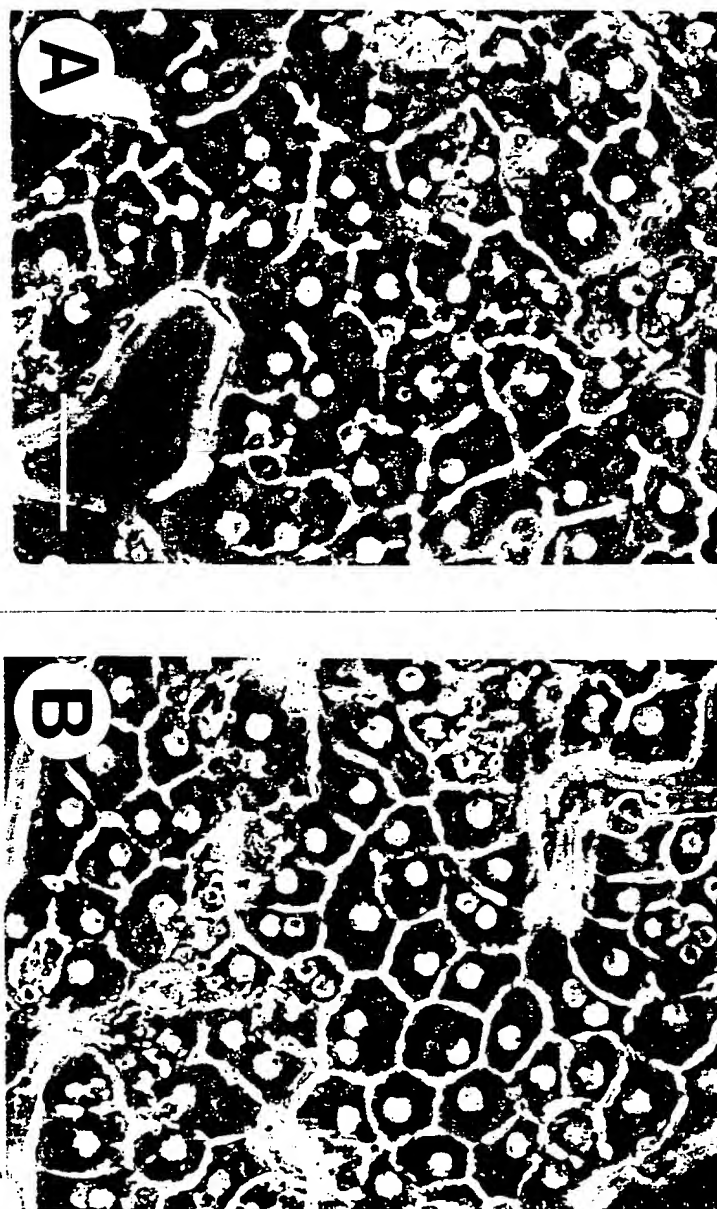


Figure 3-1. Effect of extracellular matrix configuration on hepatocyte morphology. Cultured hepatocytes maintained for 5 days on (A) a single layer of gelled collagen, or (B) between two layers of gelled collagen. Bar = 50 μ m.

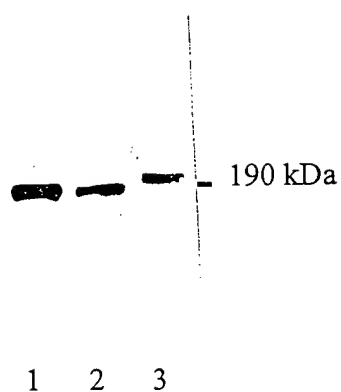


Figure 3-2. Immunoblot analysis of cMOAT in primary cultured rat hepatocytes. Lane 1 was loaded with canalicular plasma membranes isolated from rat livers (10 μ g protein); lanes 2 and 3 were loaded with crude membranes isolated from hepatocytes cultured on a gelled substratum for 2-4 hr (day-0) and in a collagen-sandwich configuration for 5 days, respectively (50 μ g protein). cMOAT was detected as a single band at 190 kDa in all membrane preparations.

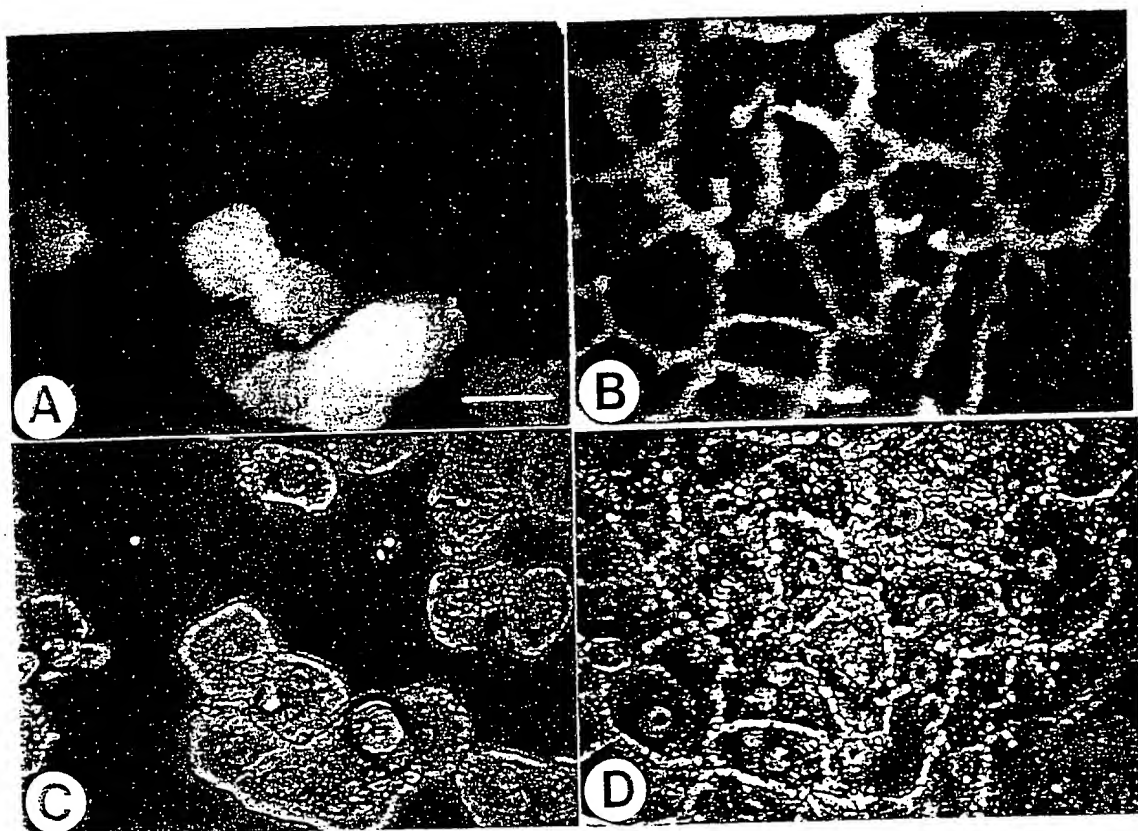


Figure 3-3. Fluorescence micrographs (A [day-0] and B [day-5]) and phase-contrast micrographs (C [day-0] and D [day-5]) of hepatocytes cultured on a gelled collagen (day-0) or in a sandwich configuration (day-5) after pretreatment with carboxydichlorofluorescein diacetate. In day-0 hepatocyte cultures, fluorescence was concentrated between a few hepatocytes (A, arrow). In day-5 hepatocyte cultures, fluorescence was exclusively localized within the bile canaliculi (B, arrow), demonstrating the functional activity of cMOAT in the canalicular membrane. Bar = 30 μ m.

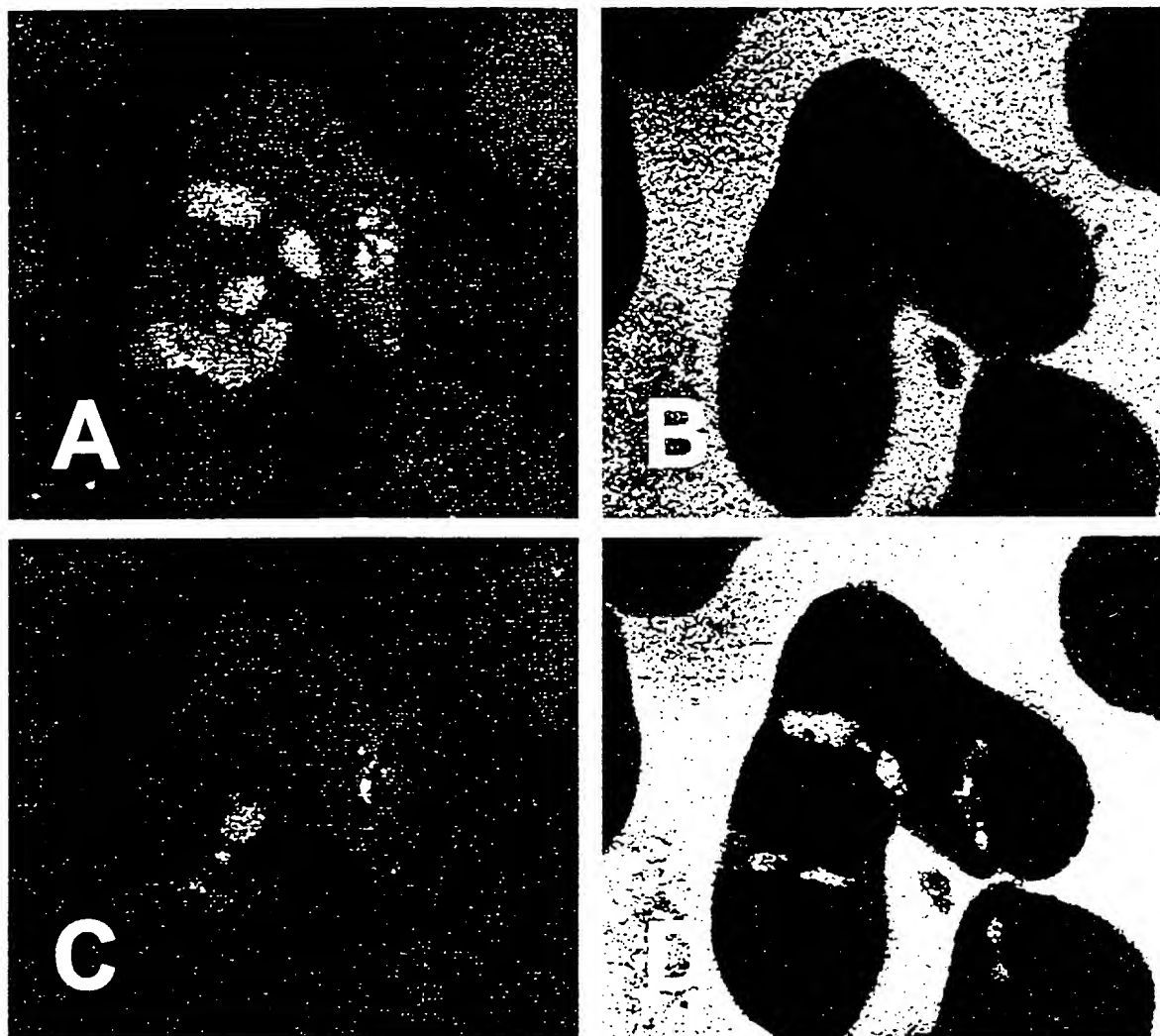


Figure 3-4. Effect of calcium modulation on the integrity of canaliculi networks in hepatocytes cultured in a sandwich configuration. Confocal images were collected with a Bio-Rad MRC-600 confocal microscope in the fluorescein (A) and rhodamine (B) channels. Day-5 sandwich-cultured hepatocytes were preloaded with carboxydichlorofluorescein and maintained in rhodamine-dextran solution in standard buffer. After rinsing with Ca^{2+} -free buffer twice, the monolayer was maintained for 5 min in Ca^{2+} -free rhodamine-dextran solution prior to collecting the images in the fluorescein (C) and rhodamine (D) channels. In Ca^{2+} -free buffer, carboxydichlorofluorescein and rhodamine-dextran were able to move into the incubation medium and bile canaliculi space, respectively, based on favorable concentration gradients. Bar = 30 μm

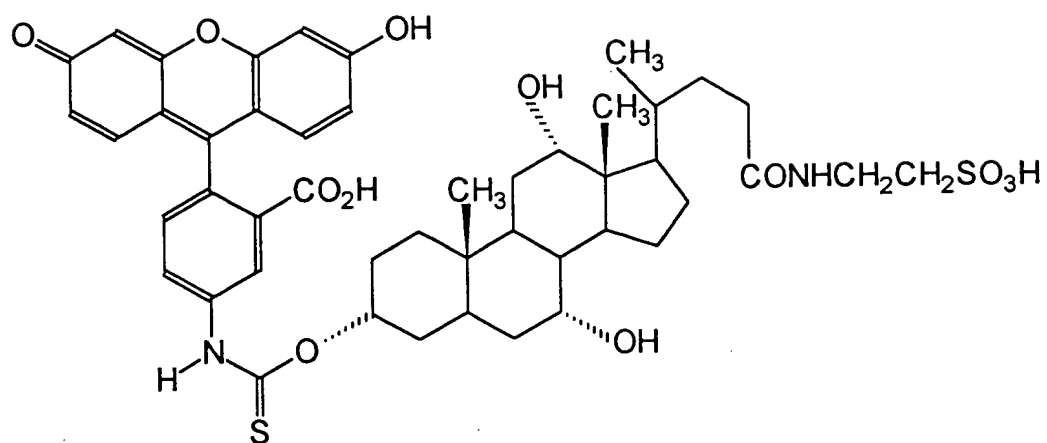


Figure 3-5. Structure of fluorescein-labeled taurocholate

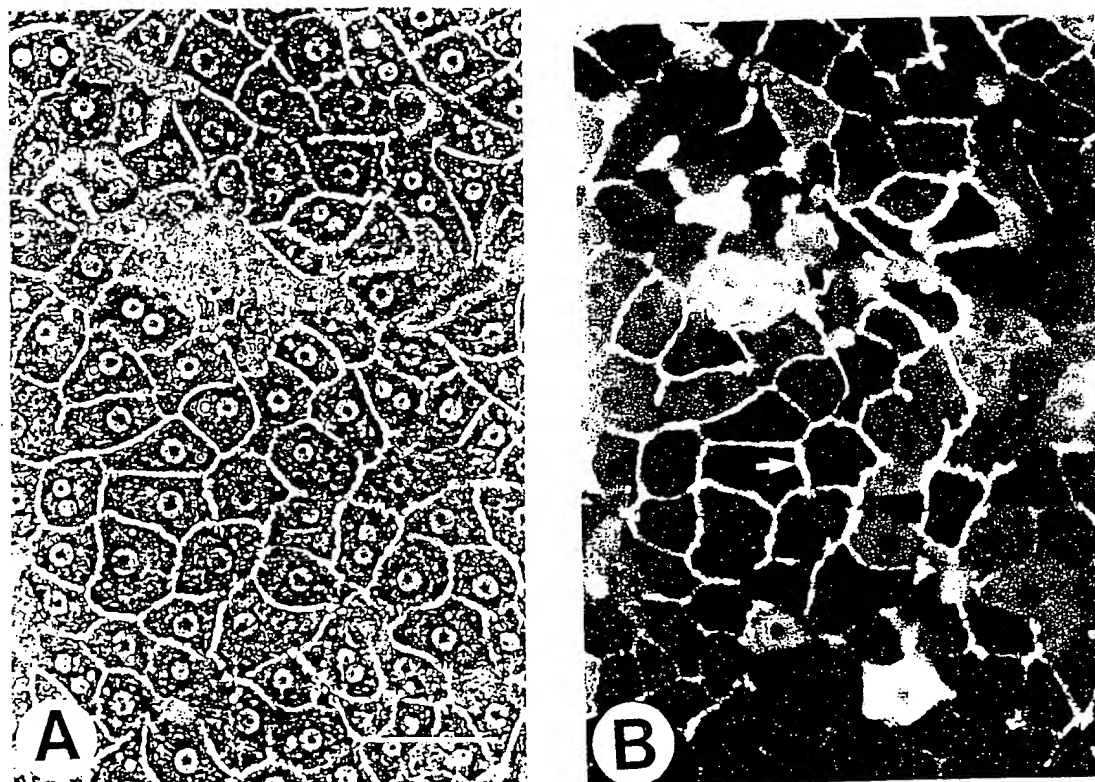


Figure 3-6. Phase contrast (A) and fluorescence image (B) of hepatocytes cultured in a sandwich configuration for 5 days after pretreatment with 1 $\mu\text{g/ml}$ fluorescein-labeled taurocholate. Fluorescence was localized within the bile canaliculi (arrow), demonstrating the functional activity of the bile acid transporter in the canalicular membrane. Bar = 50 μm .

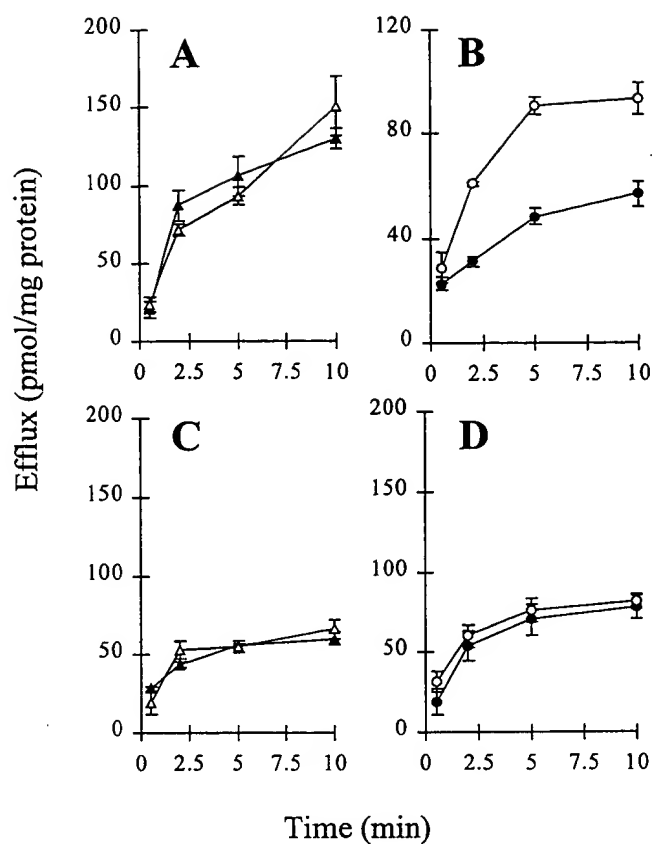


Figure 3-7. Efflux of [³H]taurocholate (A, B) and [¹⁴C]salicylate (C, D) in standard buffer (closed symbols) and Ca²⁺-free buffer (open symbols) from hepatocyte monolayers cultured for approximately 4 hr (A, C) and hepatocytes cultured in a sandwich configuration for 5 days (B, D). The hepatocytes were preincubated for 10 min in standard buffer containing 1 μ M [³H]taurocholate and 3.6 μ M [¹⁴C]salicylate, respectively, before the efflux studies were conducted.

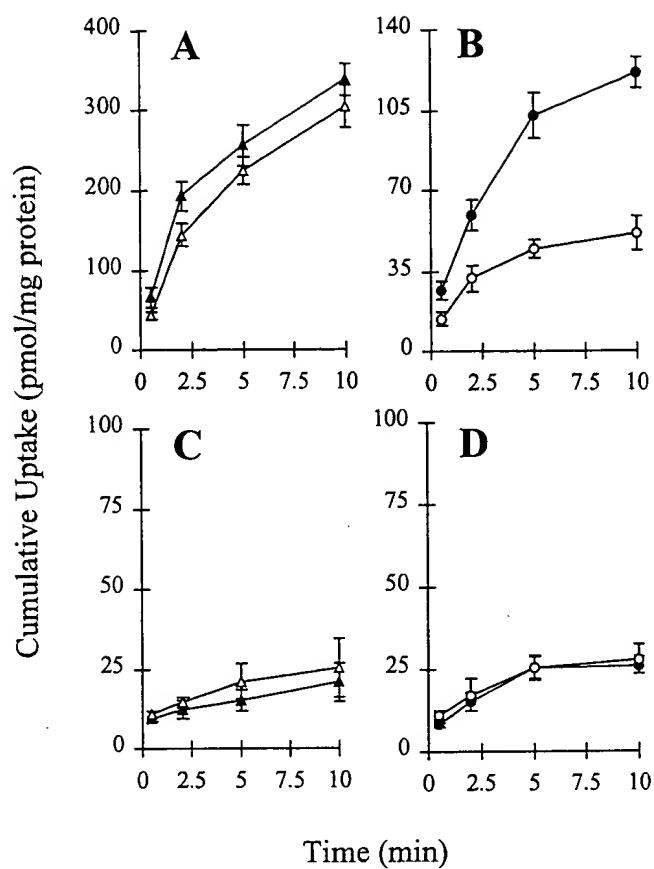


Figure 3-8. Cumulative uptake of $[^3\text{H}]$ taurocholate ($1\ \mu\text{M}$) (A, B) and $[^{14}\text{C}]$ salicylate ($1\ \mu\text{M}$) (C, D) in hepatocyte monolayers cultured for approximately 4 hr (A, C) and hepatocytes cultured in a sandwich configuration for 5 days (B, D). The hepatocytes were preincubated for 10 min in standard buffer (closed symbols) and Ca^{2+} -free buffer (open symbols), respectively, before the cumulative uptake studies were conducted.

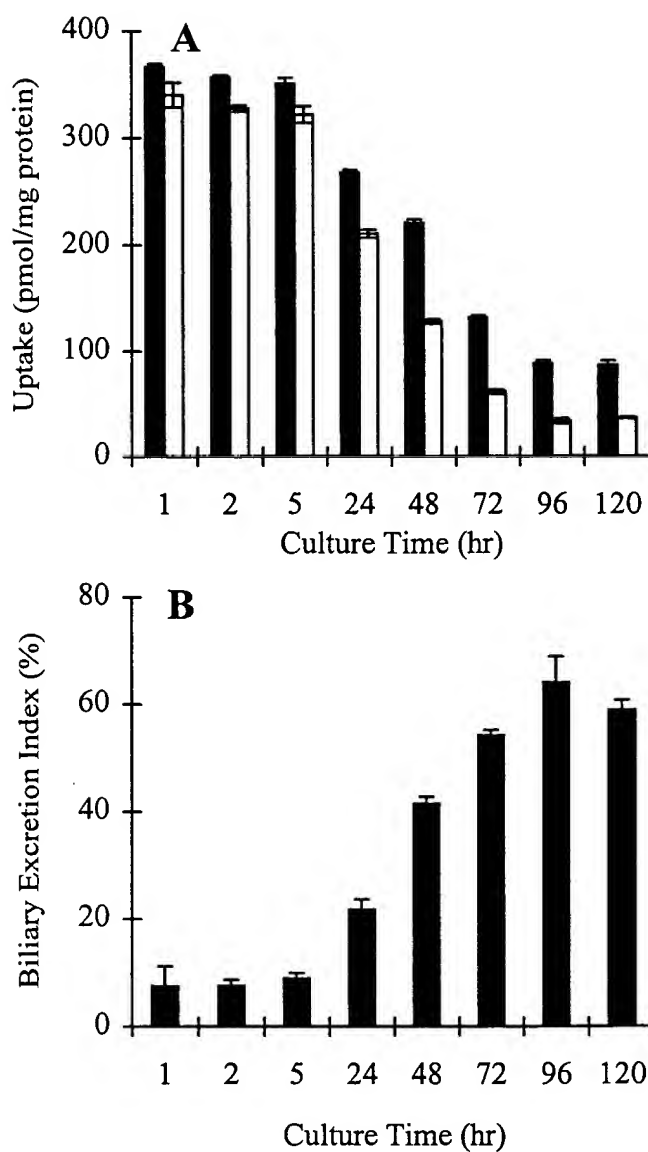


Figure 3-9. Relationship between the polarized excretory function of sandwich-cultured hepatocytes and culture time. After the cultured hepatocyte monolayers were preincubated in standard buffer (panel A: closed bars) or in Ca²⁺-free buffer (panel A: open bars) for 10 min, respectively, the cumulative uptake of 1 μ M [³H]taurocholate was measured in the monolayers at 10 min. The Biliary Excretion Index of taurocholate (panel B), calculated from panel A, increased with culture time.

CHAPTER 4

USE OF Ca^{2+} MODULATION TO EVALUATE BILIARY EXCRETION IN SANDWICH-CULTURED RAT HEPATOCYTES

This chapter has been submitted to the *Journal of Pharmacology and Experimental Therapeutics*, and is presented in the style of that journal.

ABSTRACT

Previous work has indicated that biliary excretion of a substrate in sandwich-cultured hepatocytes can be quantitated by measurement of cumulative substrate uptake in the presence and absence of extracellular Ca^{2+} . The present study was designed to examine the effects of Ca^{2+} on (1) taurocholate uptake and (2) tight junction integrity in cultured hepatocytes. Kinetic modeling was employed to characterize taurocholate disposition in the hepatocyte monolayers in the presence and absence of extracellular Ca^{2+} . The cumulative uptake of taurocholate in freshly isolated hepatocytes, which lack an intact canalicular network, was the same in the presence and absence of extracellular Ca^{2+} , demonstrating that taurocholate transport was independent of extracellular Ca^{2+} levels. Electron microscopy studies showed that Ca^{2+} depletion increased the permeability of the tight junctions to ruthenium red, demonstrating that tight junctions were the major diffusional barrier between the canalicular lumen and the extracellular space. Cell morphology and cumulative uptake studies in the monolayers indicated that Ca^{2+} depletion disrupted the tight junctions in 1-2 min. The integrity of the disrupted tight junctions was not reestablished completely after incubation in the presence of Ca^{2+} for 1 hr. The cumulative uptake of taurocholate was best described by a two-compartment model (cytosol and bile) with Michaelis-Menten kinetics for both uptake and biliary excretion. In summary, Ca^{2+} depletion does not alter hepatocyte transport properties. Ca^{2+} modulation may be a useful approach to study canalicular transport of substrates in sandwich-cultured hepatocytes.

INTRODUCTION

Accurate evaluation of hepatic disposition (including hepatic metabolism, protein binding, intracellular sequestration, and biliary excretion) is necessary in the development of clinically useful drugs, as well as for predicting the pharmacological and toxicological effects of drugs, pharmacokinetic properties in humans and drug-drug interactions. Biliary excretion of substrates is a complex process involving translocation across the sinusoidal membrane, movement through the cytoplasm, and transport across the canalicular membrane. Numerous *in vitro* systems (e.g., isolated perfused livers, isolated hepatocytes, short-term cultured hepatocyte couplets, liver plasma membrane vesicles and expressed transport proteins) have been used to investigate biliary excretion processes (Oude Elferink, *et al.*, 1995).

Cultured hepatocytes represent a potential model to study the biliary excretion of a large number of substrates. This model may allow prediction of biliary excretion in humans by utilizing human hepatocytes. Short-term (3-8 hr) cultured hepatocyte couplets have been employed to directly examine the biliary excretion of fluorescent compounds utilizing fluorescence microscopy (Graf and Boyer, 1984; 1990). However, the application of cultured hepatocyte couplets to study biliary excretion of xenobiotics is limited because the substrate must contain a fluorescent chromophore. Long-term (more than 24 hr) cultured hepatocytes have been reported to restore polarity with canalicular-like structures and to develop an asymmetrical distribution of the sinusoidal and canalicular membrane proteins (Barth and Schwarz, 1982; Maurice *et al.*, 1988; Talamini *et al.*, 1997). Although primary hepatocytes maintained under conventional culture conditions have been used to study drug metabolism and hepatotoxicity, long-term cultures of hepatocytes have not been a suitable model for

studying hepatobiliary transport due to the rapid loss of liver-specific functions, including hepatic transport properties, and failure to reestablish normal bile canalicular networks and maintain normal hepatocyte morphology (Groothuis and Meijer, 1996; LeCluyse *et al.*, 1996a).

Modifications to conventional culture conditions have resulted in dramatic improvements in the maintenance of hepatic function and longevity of hepatocyte cultures (Maher, 1988). One successful approach was to mimic the native extracellular matrix geometry by maintaining hepatocytes between two layers of a collagen gel or in a collagen-sandwich configuration (Dunn *et al.*, 1989; LeCluyse *et al.*, 1994). Maintenance of hepatocytes in a collagen-sandwich configuration prolongs hepatocyte viability and preserves liver-specific protein synthesis. Further studies demonstrated that long-term cultured hepatocytes in a collagen-sandwich configuration reestablish a bile canalicular network and show better maintenance of drug uptake and enzyme-induction potential (Sidhu *et al.*, 1993; LeCluyse *et al.*, 1996b). Recently, it has been demonstrated that Na⁺/taurocholate cotransporting polypeptide was partially maintained in hepatocytes cultured in a collagen-sandwich configuration for 4-5 days (Chapter 2). Functional activity of the canalicular bile acid transporter and the canalicular multispecific organic anion transporter also has been demonstrated in the hepatocyte monolayers cultured in a collagen-sandwich configuration (Chapter 3). Furthermore, a technique was described recently to quantitate the amount of substrate in the bile canaliculi of long-term cultured hepatocytes maintained in a sandwich configuration by determination of cumulative uptake of substrate in the presence and absence of Ca²⁺ in the incubation medium (Chapter 3). This method would allow the quantitative

assessment of hepatocyte polarization during the course of culture. More importantly, this *in vitro* model system provides a new-approach to examine biliary excretion of nonfluorescent compounds with higher efficiency and greater versatility than other existing approaches.

In a previous study, Ca^{2+} depletion was used to increase the permeability of tight junctions in hepatocyte cultures. However, the effects of Ca^{2+} depletion on the transport properties and tight junctions of collagen-sandwich hepatocyte monolayers have not been examined extensively. The primary objective of this study was to investigate further the effects of Ca^{2+} modulation on the sandwich-cultured hepatocytes. A multi-experimental approach was employed to examine the effect of Ca^{2+} depletion on (1) taurocholate uptake processes and (2) the permeability of tight junctions in the sandwich-cultured hepatocytes. A secondary objective was to develop a kinetic model to describe the cumulative uptake data in sandwich-cultured hepatocyte monolayers and to examine the transport processes in this *in vitro* model. The results from the present study further demonstrate that hepatocytes cultured in a collagen-sandwich configuration represent a useful *in vitro* system which can be utilized to study hepatobiliary disposition of xenobiotics.

MATERIALS AND METHODS

Chemicals. Taurocholate, dexamethasone, ruthenium red, Hanks' balanced salt solution, and Ca^{2+} , Mg^{2+} -free Hanks' balanced salt solution were purchased from Sigma Chemical Co. (St. Louis, MO). [^3H]Taurocholate (3.4 Ci/mmol, purity > 97%) and [^3H]inulin (1.3 Ci/mmol, purity > 97%) were obtained from Dupont New England Nuclear (Boston, MA). Collagenase (type I, class I) was obtained from Worthington Biochemical Corp. (Freehold, NJ). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum and insulin were purchased from Gibco (Grand Island, NY). Rat tail collagen (type I) was obtained from Collaborative Biomedical Research (Bedford, MA). All other chemicals and reagents were of analytical grade and were readily available from commercial sources.

Animals. Male Wistar rats (250-280 g) from Charles River Laboratory (Raleigh, NC) were used as liver donors. Rats were housed individually in stainless-steel cages in a constant alternating 12-hr light and dark cycle at least 1 week before the study was performed, and were fed ad libitum until use. All procedures were approved by the Institutional Animal Care and Use Committee.

Hepatocyte Isolation. Hepatocytes were isolated with a two-step perfusion method as reported previously (Chapter 2). Rats were anesthetized with ketamine and xylazine (60 and 12 mg/kg i.p., respectively) prior to portal vein cannulation. The liver was perfused *in situ* with oxygenated Ca^{2+} -free Krebs-Henseleit bicarbonate buffer containing 5.5 mM glucose for 10 min at 37°C followed by perfusion with Krebs-Henseleit bicarbonate buffer containing collagenase type I (0.5 mg/ml) for 10 min. The hepatic capsule was removed with forceps. The hepatocytes were released by shaking the liver gently in 100 ml DMEM. The released

cells were filtered through a sterile nylon mesh (70- μ m). The hepatocyte suspensions were centrifuged at 50 \times g for 3 min. The cell pellet was resuspended in 25 ml DMEM and an equal volume of 90% isotonic Percoll (pH 7.4); the resulting cell suspension was centrifuged at 150 \times g for 5 min. The pellet was resuspended in 50 ml DMEM and the cell suspensions were combined into one tube followed by centrifugation at 50 \times g for 3 min. Hepatocyte viability was determined by trypan blue exclusion. Only those hepatocyte preparations with viability greater than 90% were utilized for further studies.

Uptake of Taurocholate in Isolated Hepatocytes. Taurocholate uptake studies in freshly prepared hepatocyte suspensions were conducted with a modified method described by Studenberg and Brouwer (1993). Hepatocytes were suspended in ice-cold standard buffer to obtain a cellular protein concentration of approximately 2.0 mg/ml and stored in an ice bath. An aliquot of 4 ml of the hepatocyte suspension was centrifuged at 50 \times g for 2 min. The resulting pellet was suspended in 4 ml of Hanks' balanced salt solution (standard buffer) or Ca^{2+} , Mg^{2+} -free Hanks' balanced salt solution with 1 mM EGTA (Ca^{2+} -free buffer), and incubated at 37°C with 95% O_2 and 5% CO_2 for 10 min. After addition of 0.1-ml [^3H]taurocholate to the suspended hepatocytes, 0.1-ml aliquots were taken at designated times, and added to 0.4-ml polyethylene microfuge tubes containing 0.05-ml silicone oil (diluted to a density of 1.03 with mineral oil) layered on top of 0.05 ml of 3 M KOH. The samples were centrifuged for 10 seconds in a table-top microfuge (Beckman Instruments, Inc., Fullerton, CA). The amount of [^3H]taurocholate taken up into the hepatocytes was determined by cutting the tubes at the oil interface, placing the cell lysate in a scintillation vial with 5-ml cocktail (Atomflow, Packard) and analyzing by liquid scintillation

spectrometry. Adherent fluid volume on the surface of hepatocytes was determined with [^3H]inulin.

Preparation of Culture Dishes. Plastic culture dishes (60 mm) were precoated with rat tail collagen at least 1 day prior to preparing the hepatocyte cultures. To obtain a gelled collagen substratum, ice-cold neutralized collagen solution (0.1 ml, 1.5 mg/ml, pH 7.4) was spread onto each culture dish. Freshly coated dishes were placed at 37°C in a humidified incubator for approximately 1 hr to allow the matrix material to gel, followed by addition of 3 ml DMEM to each dish and storage in a humidified incubator.

Cultured Rat Hepatocytes. Hepatocyte suspensions were prepared with DMEM containing 5% fetal calf serum, 1 μM dexamethasone and 4 mg/L insulin. Hepatocyte suspensions were added to the pre-coated dishes at a density of 2×10^6 cells/60-mm dish. Approximately 1 hr after plating the cells, the medium was aspirated and 3-ml fresh DMEM was added. For hepatic transport studies, hepatocytes that had been seeded for 3-5 hr without collagen overlay were defined as day-0 or short-term cultured hepatocytes.

To prepare sandwich-cultured hepatocytes, neutralized collagen solution (0.1 ml, 1.5 mg/ml, pH 7.4) was added to the monolayers 24 hr after the cells were seeded. Cultures with collagen overlay were incubated for 45 min at 37°C in a humidified incubator to allow the collagen to gel before addition of DMEM. Medium was changed on a daily basis until the fourth day after the cells were seeded. These hepatocytes were referred to as 96-hr or long-term cultured hepatocytes.

Electron Microscopy. Hepatocytes cultured on Permanox dishes in a sandwich configuration were incubated in standard buffer or Ca^{2+} -free buffer for 10 min at 37°C, and

then fixed in a ruthenium red (0.25%)/glutaraldehyde (1.25%) solution for 1 hr at room temperature. After removal of the primary fixative solution, the cells were rinsed three times at room temperature in 0.1 M sodium cacodylate buffer. A solution of osmium tetroxide (1.3%)/ruthenium red (0.2%)/cacodylate (0.7M) was applied and the cells were allowed to post fix for 1 hour. Subsequently cells were rinsed three times with sodium cacodylate buffer, dehydrated and embedded in Spurr resin. The embedded cultures were removed from the Permax dishes and the area of interest selected for re-embedding. Semi-thin sections were cut and stained with toluidine blue and examined prior to cutting ultra-thin sections. Ultra-thin sections were placed on copper grids, and examined unstained with a Jeol 100C Transmission Electron Microscope (Jeol, Tokyo, Japan).

Uptake Studies in Sandwich-Cultured Hepatocytes. Hepatocytes cultured in a collagen-sandwich configuration were incubated in 3 ml standard buffer or Ca^{2+} -free buffer at 37°C . After removing the incubation buffer, uptake was initiated by addition of 3 ml standard buffer containing [^3H]taurocholate to each dish. After incubation for designated times, cumulative uptake was terminated by aspirating the incubation solution and rinsing 4 times with 3 ml ice-cold standard buffer to remove extracellular substrate. After washing, 2 ml of 1% Triton X-100 solution was added to culture dishes to lyse cells by shaking the dish on a shaker for 20 min at room temperature. An aliquot (1 ml) of lysate was analyzed by liquid scintillation spectrometry. Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA) was used to determine the protein concentration in the culture extracts using bovine serum albumin as standard. Triton X-100 (1%) did not interfere with the protein assay. All values for taurocholate uptake into cell monolayers were corrected for nonspecific binding to

the collagen by subtracting taurocholate uptake determined in the appropriate control dishes in the absence of cells as described previously (Chapter 2).

Model Development. The average cumulative uptake vs. time data for taurocholate (1-100 μM) were used in model development. Differential equations corresponding to a series of models shown schematically in Figure 4-5 with combinations of first-order and Michaelis-Menten parameters provided in Table 4-1, were solved simultaneously with the nonlinear least-squares regression program WinNonlin (version 1.1, Scientific Consulting Inc., Apex, NC). Models incorporated two different compartment structures (Figure 4-5). In Models #1-5, cell and bile canaliculi were localized in the same compartment. In Models #6-17, cell and bile canaliculi were localized in separate compartments. Each model was based on two assumptions: (1) pre-incubation in Ca^{2+} -free buffer did not influence the membrane transport processes, and (2) the translocation processes were unidirectional. Model selection and assessment of goodness-of-fit were based on Akaike's Information Criterion (AIC; Akaike, 1976), the degree of co-linearity of parameters, the standard error of parameter estimates, the degree of bias in residual error, and visual inspection of the generated curves relative to the data. A weighing scheme of $1/Y$ was used for all fitting procedures.

Data analysis. Uptake data were normalized to the protein content and expressed as mean \pm SD from 3-4 separate preparations of hepatocytes. Differences in cumulative uptake between two experimental conditions were analyzed by Student's *t*-test. A *P* value of < 0.05 was considered significant.

RESULTS

— *Uptake of Taurocholate in Freshly Isolated Hepatocytes.* The effects of Ca^{2+} on taurocholate cumulative uptake were examined in freshly isolated hepatocytes incubated in standard or Ca^{2+} -free buffer for 10 min prior to the addition of [^3H]taurocholate. Taurocholate uptake at 4°C was 2-7% of cumulative uptake at 37°C , as expected for an active transport process (Figure 4-1). The initial uptake rates of taurocholate in standard buffer (6.53 ± 0.13 nmol/min/mg protein) and in Ca^{2+} -free buffer (5.91 ± 0.88 nmol/min/mg protein) were not significantly different ($p > 0.05$). The cumulative uptake of taurocholate at 10 min in freshly isolated hepatocytes was not significantly different in the presence and absence of extracellular Ca^{2+} ($p > 0.05$).

Electron Microscopy and Ruthenium Red Staining. Electron microscopy studies utilizing ruthenium red staining were employed to determine directly whether disruption of the integrity of canalicular networks in sandwich-cultured hepatocytes by Ca^{2+} depletion was secondary to the increased permeability of the tight junctions around the canaliculi. For sandwich-cultured hepatocyte monolayers incubated in standard buffer, ruthenium red staining was visible on the plasma membranes that were in direct contact with the collagen gel layer and along intercellular membranes, but was not present on the membranes lining the canalicular space (Figure 4-2A). Ruthenium red staining also was visible on the basolateral membranes of sandwich-cultured hepatocytes incubated in Ca^{2+} -free buffer. However, in contrast to the hepatocyte monolayers incubated in the presence of Ca^{2+} , ruthenium red staining was present on the membranes lining the canalicular space (Figure 4-2B). These observations directly demonstrated that Ca^{2+} depletion disrupted the barrier function of the

tight junctions between the canalicular and extracellular spaces. In addition, the canalicular spaces between adjacent hepatocytes incubated in Ca^{2+} -free buffer were smaller in diameter compared to the hepatocytes incubated in standard buffer.

Effects of Ca^{2+} on Canalicular Morphology and Taurocholate Uptake. To examine the effects of Ca^{2+} on the morphology of sandwich-cultured hepatocytes, hepatocyte monolayers maintained in a sandwich configuration for 4 days were incubated in Ca^{2+} -free buffer for 10 min followed by incubation in standard buffer. The long-term sandwich-cultured hepatocytes formed dilated canaliculi between adjacent hepatocytes (Figure 4-3A). After incubation of the monolayers for 10 min in Ca^{2+} -free buffer, the size of the canaliculi was reduced drastically (Figure 4-3B). Subsequently, the monolayers were incubated in standard buffer to determine if the contracted canaliculi could be re-dilated. Canalicular size did not change considerably after incubation of the monolayers in standard buffer for 10 min (Figure 4-3C), but after incubation in standard buffer for 60 min, the majority of canaliculi had re-dilated (Figure 4-3D). However, the apparent size of all the canaliculi was not as great as prior to incubation in Ca^{2+} -free buffer.

The integrity of the tight junctions in the sandwich-cultured hepatocyte monolayers also was evaluated by examining the time-course of the effects of Ca^{2+} depletion on cumulative uptake of the model substrate taurocholate. The 10-min cumulative uptake of [^3H]taurocholate was determined in standard buffer after the hepatocyte monolayers were incubated in Ca^{2+} -free buffer for designated times (Figure 4-4A). After the hepatocyte monolayers were incubated in Ca^{2+} -free buffer for 1-2 min, the 10-min cumulative uptake of taurocholate was approximately 60% of the cumulative uptake in the hepatocyte monolayers

that had not been incubated (0 min) ($p < 0.001$). The 10-min cumulative uptake decreased to approximately 40% and 30% of control values after incubation in Ca^{2+} -free buffer for 10 min and 60 min, respectively ($p < 0.001$). The 10-min cumulative taurocholate uptake in hepatocytes pre-incubated in Ca^{2+} -free buffer for 1 min was significantly higher than hepatocytes pre-incubated in Ca^{2+} -free buffer for 10 min ($p < 0.05$). However, uptake in the hepatocytes pre-incubated in Ca^{2+} -free buffer for 2, 5, 20, or 60 min failed to show significant differences in 10 min cumulative taurocholate uptake compared to hepatocytes pre-incubated in Ca^{2+} -free buffer for 10 min.

A second series of experiments was conducted to investigate whether the decrease in cumulative uptake of taurocholate in the sandwich-cultured hepatocytes incubated in Ca^{2+} -free buffer could be restored. The monolayers were first incubated in Ca^{2+} -free buffer for 10 min to disrupt the tight junctions, and then incubated in standard buffer for designated times prior to determining a 10-min cumulative uptake in standard buffer containing 1 μM [^3H]taurocholate (Figure 4-4B). The 10-min cumulative uptake of taurocholate increased as incubation times increased in standard buffer. Compared to the monolayers that were not incubated in standard buffer prior to initiation of uptake (0 min), 5-min incubation of the monolayers in standard buffer did not alter the cumulative uptake, but 10, 20, and 60-min incubation in standard buffer significantly increased the cumulative uptake ($p < 0.05$). However, the cumulative taurocholate uptake in the hepatocyte monolayers that were incubated for 60 min in standard buffer was still ~20% less than the uptake in hepatocytes that had not been incubated in Ca^{2+} -free buffer (control, $p < 0.01$).

Kinetic Analysis of Cumulative Uptake in Hepatocyte Monolayers. To characterize the kinetic processes involved in basolateral uptake and canalicular excretion of substrate in the hepatocyte monolayers cultured for 4 days in a sandwich configuration, cumulative uptake studies of taurocholate (1-100 μM) in standard buffer were undertaken in the monolayers which had been incubated in standard buffer or Ca^{2+} -free buffer at 37°C for 10 min (Figure 4-6). Seventeen different models were employed to fit the cumulative uptake data (Figure 4-5, Table 4-1) in order to define an appropriate model to describe the cumulative uptake kinetics. All models were of full rank, indicating that there were sufficient data to precisely estimate all the parameters. The condition number of the matrix of partial derivatives was less than 10^6 , suggesting a low degree of co-linearity between parameters in the models. According to AIC and visual examination, Model 13 provided the best description of the cumulative uptake of taurocholate in the sandwich-cultured hepatocytes (Table 4-2, Figure 4-6). The differential equations corresponding to Model 13 are:

$$\frac{dX_{\text{standard}}}{dt} = \frac{V_{ma} \cdot C}{K_{ma} + C} - K_{e4} \cdot (X_{\text{standard}} - X_{\text{Ca}^{2+}\text{-free}}) \quad (1)$$

$$\frac{dX_{\text{Ca}^{2+}\text{-free}}}{dt} = \frac{V_{ma} \cdot C}{K_{ma} + C} - \frac{V_{mb} \cdot X_{\text{Ca}^{2+}\text{-free}}}{K_{mb} + X_{\text{Ca}^{2+}\text{-free}}} \quad (2)$$

where X_{standard} is the cumulative amount of taurocholate taken up in standard buffer, $X_{\text{Ca}^{2+}\text{-free}}$ is the cumulative amount of taurocholate taken up in Ca^{2+} -free buffer, C is the taurocholate concentration in the incubation buffer, V_{ma} is the maximal velocity for uptake, K_{ma} is the apparent Michaelis-Menten constant for uptake, K_{e4} is the first-order rate constant for elimination from the bile compartment in standard buffer, V_{mb} is the maximal velocity for

canalicular (biliary) excretion, and K_{mb} is the apparent Michaelis-Menten constant for canalicular (biliary) excretion. Kinetic parameter estimates for Model 13 are presented in Table 4-3.

Comparison of Models 1, 2, and 3 with Models 8, 11, and 14, respectively, demonstrated that separation of the intracellular space and the bile canalicular space into two different compartments provided a better fit to the data, as indicated by lower AIC values (Table 4-2). In addition, in standard buffer, first-order elimination from the bile compartment (E) described the cumulative uptake data better than first-order elimination from the cell compartment (D) (Models 7, 10, and 13 vs. Models 6, 9, and 12, respectively), or first-order elimination from both the cell and bile compartments (E and D) (Models 7, 10, and 13 vs. Models 8, 11, and 14, respectively). Furthermore, in Ca^{2+} -free buffer, first-order elimination from the bile compartment (E) described the data better than a Michaelis-Menten process (Model 13 vs. Model 16). Basolateral uptake of taurocholate (A) was described better by a Michaelis-Menten process as compared to a first-order process (Models 9, 10, and 11 vs. Models 6, 7, and 8, respectively) based on smaller AIC values and visual examination. Although the total sum of square residuals between the observed and model predicted data was slightly smaller in Model 15 compared to Model 13 (506.7 vs. 507.2), the AIC of Model 15 was greater than Model 13 (385.7 vs. 383.7). These results suggested that a Michaelis-Menten basolateral uptake process in parallel with a first-order uptake process did not improve the fit significantly. Elimination from the cell compartment (F), representing biliary excretion across the canalicular membrane, was described better by a Michaelis-Menten process as compared to a first-order process (Models 12, 13, and 14 vs. Models 9, 10, and 11,

respectively), or a first-order process in parallel with a Michaelis-Menten elimination process (Model 13 vs. Model 17).

DISCUSSION

In the present study, a variety of techniques have been used to investigate the effects of Ca^{2+} depletion on the transport properties and tight junctions of hepatocytes cultured in a sandwich configuration. The results indicate that: (1) Ca^{2+} depletion does not alter taurocholate transport; (2) Ca^{2+} depletion increases the permeability of tight junctions thus disrupting the barrier between the canalicular lumen and the extracellular space; (3) integrity of the disrupted tight junctions cannot be reestablished completely by incubation in the presence of Ca^{2+} for 1 hr; and (4) cumulative uptake of taurocholate in the hepatocyte monolayers can be described by a two-compartment model with nonlinear uptake and nonlinear biliary excretion processes.

Hepatocytes cultured in a collagen-sandwich configuration for 6 days form complete junctional complexes composed of a tight junction, intermediate junction and desmosomes (LeCluyse *et al.*, 1994). Recently, Talamini *et al.* (1997) demonstrated the existence of junctional protein, uvomorulin (E-cadherin), in hepatocytes cultured in a sandwich configuration. Hepatocytes cultured in a sandwich configuration for 4-5 days consist of two compartments: the canalicular lumen and the extracellular space. Previous studies (Chapter 3) using confocal fluorescence microscopy demonstrated indirectly that the barrier between the canalicular lumen and the incubation medium was disrupted after Ca^{2+} depletion, allowing substrates to translocate between these two compartments depending on favorable concentration gradients. The present studies provide direct evidence that disruption of the integrity of the bile canalicular lumen by Ca^{2+} depletion is caused by enhanced permeability of tight junctions in the hepatocyte cultures.

Localization of ruthenium red staining in the presence and absence of extracellular Ca^{2+} was utilized to directly examine the barrier function of tight junctions in the monolayers. Ruthenium red does not penetrate intact plasma membranes, but binds to intercellular membranes; and will penetrate to the level of the tight junction in non-leaky epithelia (Mullin *et al.*, 1997; van Deurs *et al.*, 1996). In this study, the electron-dense dye ruthenium red was clearly identified on the basolateral membranes of sandwich-cultured hepatocyte monolayers, but was excluded from the canalicular lumen. In contrast, in the hepatocyte monolayers incubated in Ca^{2+} -free buffer prior to fixation and staining, ruthenium red was bound to all regions of the plasma membrane including the lining of the canaliculi. This indicated that Ca^{2+} depletion lead to disruption of the tight junctions, allowing access of ruthenium red to the interior of the canaliculi.

In addition, electron microscopy studies indicated that the size of canaliculi appeared to be smaller in diameter in the sandwich-cultured hepatocyte monolayers incubated in Ca^{2+} -free buffer as compared to those in standard buffer. This observation also was apparent in monolayers viewed by light microscopy, in which the canaliculi “collapsed” after the monolayers were exposed to Ca^{2+} -free buffer (Figure 4-3B). The maintenance of functional tight junctions around a canaliculus may result in the observed dilation of the canaliculi. When the junctions were disrupted, the contents of the canaliculi were released into the extracellular medium causing the canaliculi to decrease in size.

The present study also demonstrated that taurocholate uptake is independent of the extracellular Ca^{2+} concentration. As described previously (Chapter 3), the cumulative uptake of taurocholate in hepatocytes cultured in a sandwich configuration in standard buffer

represented taurocholate in the intracellular spaces and the bile canalicular lumen, while cumulative uptake of taurocholate in Ca^{2+} -free buffer only represented the intracellular taurocholate due to disruption of the tight junctional barrier in the monolayers. Thus, taurocholate in the bile canalicular lumen can be quantitated by measuring the difference in cumulative uptake in standard and Ca^{2+} -free buffer. One critical assumption in this calculation is that Ca^{2+} depletion does not modulate taurocholate transport, including uptake and excretion. Freshly isolated hepatocytes that lose hepatic architecture and intact canalicular tight junctions (Graf and Boyer, 1990) represent an ideal model to investigate this assumption. The present study demonstrated that the initial uptake rate of taurocholate as well as the 10-min cumulative uptake of taurocholate was not statistically different in the isolated hepatocytes incubated in standard and Ca^{2+} -free buffer ($p > 0.05$), suggesting that Ca^{2+} modulation did not alter taurocholate transport processes. This result was consistent with previous observations that hepatic uptake and secretion of taurocholate in isolated hepatocytes are not dependent on extracellular Ca^{2+} (Anwer and Clayton, 1985).

Previous confocal fluorescence microscopy studies in hepatocyte monolayers demonstrated that the fluorescence intensity of canalicular networks in the monolayers after incubation with carboxydichlorofluorescein diacetate attenuated considerably after exposure to Ca^{2+} -free buffer for 1-2 min, and the fluorescent canalicular networks disappeared completely in approximately 5 min (Chapter 3). These observations suggest that integrity of the tight junctions was disrupted by Ca^{2+} depletion relatively quickly. In the present study, the relationship between the incubation time of sandwich-cultured hepatocytes in Ca^{2+} -free buffer and cumulative taurocholate uptake was examined as a measure of the extent of

disruption of the tight junction integrity. Consistent with fluorescence studies, cumulative taurocholate uptake decreased significantly after exposure of the hepatocyte monolayers to Ca^{2+} -free buffer for 1-2 min. Thereafter, the cumulative uptake continued to decrease during the course of study (up to 60 min, $p < 0.001$). Rapid disruption of the barrier function of tight junctions has been described by Citi (1992) in cultured Madin-Darby canine kidney cells. Removal of Ca^{2+} by exposure to Ca^{2+} -free buffer with 1 mM EGTA reduced the transepithelial electrical resistance within 5 min. Thus, modulation of tight junction permeability by extracellular Ca^{2+} appeared to be similar between primary cultured hepatocytes and Madin-Darby canine kidney cells. In order to increase the permeability of tight junctions, but to avoid the potential interfering effects of prolonged Ca^{2+} depletion on cellular function, 10-min incubations of hepatocyte monolayers in Ca^{2+} -free buffer were employed in transport studies.

Although Ca^{2+} depletion did not interfere with uptake of the model substrate taurocholate, it is possible that Ca^{2+} depletion may interfere with the uptake properties of other substrates. Therefore, all cumulative uptake studies were conducted in standard buffer to prevent potential interfering effects of Ca^{2+} depletion on substrate transport in the hepatocyte monolayers. This approach was based on the assumption that the functional integrity of tight junctions could not be reestablished during the short duration of transport studies. In order to test this hypothesis, canalicular morphology and cumulative taurocholate uptake were examined at designated times during incubation in standard buffer after monolayers were incubated for 10 min in Ca^{2+} -free buffer. Canalicular size did not appear to change noticeably during exposure of the monolayers to standard buffer for 10 min (Figure 4-

3C). However, the canaliculi dilated considerably after incubation in standard buffer for 1 hr, although they were not as large as canaliculi that had not been exposed to Ca^{2+} -free buffer (control). Consistent with the changes observed in bile canalicular morphology, the 10-min cumulative taurocholate uptake in the hepatocyte monolayers that had been incubated in Ca^{2+} -free buffer gradually increased after incubation in standard buffer. However, cumulative taurocholate uptake remained significantly lower than the control values, even after incubation in standard buffer for 60 min ($p < 0.01$). These results suggested that the integrity of the disrupted tight junctions is reestablished slowly by incubation in standard buffer. All substrate uptake studies were completed within 10 min of modulation of the extracellular Ca^{2+} concentrations. Thus, quantitation of substrate biliary excretion in the sandwich-cultured hepatocytes should not be influenced by the reestablishment of tight junction integrity.

To further examine the effects of Ca^{2+} on the transport properties of sandwich-cultured hepatocytes, and to examine the utility of this *in vitro* model to study hepatobiliary disposition, kinetic modeling was utilized to analyze taurocholate cumulative uptake in the monolayers pre-incubated in standard buffer or Ca^{2+} -free buffer. Taurocholate disposition in sandwich-cultured hepatocytes involves multiple kinetic processes, including uptake across the basolateral membrane and excretion across the canalicular membrane. More than one kinetic process may be responsible for taurocholate translocation across each membrane domain. Parameter estimates obtained from fitting kinetic models to taurocholate uptake-time data in sandwich-cultured hepatocyte monolayers may reveal information obscured by conventional mass-balance analysis (Studenberg and Brouwer, 1993; Booth *et al.*, 1996). All

models were based on the assumption that activity of the membrane transporters was the same in hepatocyte monolayers pre-incubated in standard buffer or Ca^{2+} -free buffer, and each kinetic process was unidirectional. The validity of the first assumption was demonstrated for taurocholate in the cumulative uptake studies in freshly isolated hepatocytes described above. The second assumption was considered valid based on previous studies where taurocholate uptake and efflux from sandwich-cultured hepatocytes was negligible at 4°C , indicating that simple diffusion across the hepatocyte cell membrane is negligible (Chapter 1).

Several interesting issues are apparent after examination of the model structure and parameter estimates. The fact that a two-compartment model (cell compartment and bile compartment) described the cumulative uptake data better than a one-compartment model is consistent with observations from confocal fluorescence microscopy studies (Chapter 2) and electron microscopy studies discussed above. In the two-compartment modeling analysis, the amount of taurocholate localized in the cytosol was assumed to be the same in the monolayers pre-incubated in standard or Ca^{2+} -free buffer, and equal to the cumulative taurocholate uptake in the hepatocyte monolayers pre-incubated in Ca^{2+} -free buffer. This is based on the assumptions discussed previously that Ca^{2+} depletion does not modulate substrate disposition in the sandwich-cultured hepatocytes other than by increasing the permeability of tight junctions, and that the integrity of the disrupted tight junctions could not be reestablished during a 10-min incubation in the presence of Ca^{2+} .

The cumulative uptake of taurocholate in sandwich-cultured hepatocyte monolayers was described best by a model (Model 13) in which the monolayers were composed of cell and bile compartments in standard buffer and only a cell compartment in Ca^{2+} -free buffer.

Taurocholate uptake was described best by a Michaelis-Menten kinetic process ($K_m = 28.0 \pm 3.6 \mu\text{M}$). This value was close to the range of K_m values (30-50 μM ; Boyer and Meier, 1990) for taurocholate uptake in rat hepatic sinusoidal membrane vesicles. V_{\max} values for taurocholate uptake in the present study were greater than the values determined in a previous study (Chapter 1). Taurocholate uptake in hepatocytes is mediated predominantly by Na^+ /taurocholate cotransporting polypeptide and to a lesser extent by a Na^+ /independent organic anion transporter (Zimmerli *et al.*, 1989; Oude Elferink *et al.*, 1995). In the present study, addition of a parallel first-order uptake process to the Michaelis-Menten equation slightly improved the fit based on the sum of square residuals, however, the improved fit was not statistically significant. In previous studies (Chapter 1), kinetic analysis of the initial rate of taurocholate uptake in hepatocytes cultured in a sandwich configuration was described best by a Michaelis-Menten process in parallel with a first-order process. These apparent differences may be due to the fact that the concentration range of taurocholate employed in the present study (1-100 μM) was lower than in previous work (1-200 μM).

The elimination of taurocholate from the sandwich-cultured hepatocyte cell compartment in the presence of Ca^{2+} -free buffer represents the biliary excretion process in the monolayers. A Michaelis-Menten kinetic process best described the biliary excretion data, suggesting that a carrier-mediated elimination process was involved in canalicular excretion, as demonstrated previously (Muller *et al.*, 1991; Stieger *et al.*, 1992). The estimated maximal velocity for taurocholate biliary excretion was $1.82 \pm 0.36 \text{ nmol/min per mg}$ of cellular protein. Considering protein content for liver tissue is 0.20 mg per mg of liver (Seglen, 1976), the estimated maximal taurocholate secretion by normal rat liver would be

approximately 364 nmol/min per g of liver. This value is consistent with the maximal excretion rates reported for bile salts (170-350 nmol/min per g of liver; Stieger *et al.*, 1992; Klos *et al.*, 1979; Yousef *et al.*, 1987). Because the kinetic modeling analysis of the biliary excretion process was based on total mass in cytosol, the estimated Michaelis-Menten constant (expressed as nmol per mg of cellular protein) may not be readily comparable to values generated from other experimental systems, which are expressed commonly as concentration (μM).

The modeling analysis in this study suggested that first-order elimination of taurocholate occurred from the bile compartment but not the cell compartment when the sandwich-cultured hepatocyte monolayers were incubated in standard buffer with taurocholate. As expected, first-order elimination directly from the cell compartment should be negligible because simple diffusion of taurocholate across the canalicular membrane is negligible, as discussed previously. First-order elimination from the bile compartment in standard buffer presumably represents an intercellular pathway of elimination. The canalicular lumen undergoes cycles of contraction and dilation, which cause the expulsion of bile contents *in vivo* and in cultured hepatocytes (Phillips *et al.*, 1982; Watanabe *et al.*, 1991). Alternatively, bile motility may be due to the noncontractile collapse of canaliculi in response to secretory pressure, resulting in rupture of the canaliculi (Boyer, 1987; Graf and Boyer, 1990). The translocation of bile from bile canaliculi into the medium via an apparent first-order elimination process from the bile compartment is consistent with proposed mechanisms of bile flow. Kinetic modeling of the cumulative uptake of substrates in

sandwich-cultured hepatocytes may be a useful tool to identify kinetic processes and rate-limiting steps involved in substrate translocation into and out of hepatocytes.

In summary, results from this study directly demonstrate that tight junctions are the diffusional barrier between the bile canalicular lumen and the extracellular space in sandwich-cultured hepatocytes. This barrier can be disrupted rapidly and reversibly by depletion of extracellular Ca^{2+} without altering taurocholate transport. Kinetic modeling analysis indicates that taurocholate uptake and biliary excretion occur via carrier-mediated transport processes. Hepatocytes cultured in a sandwich configuration represent a useful *in vitro* model system which may be utilized to study hepatobiliary disposition of compounds.

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Table 4-1. Rate Constants Associated With Cumulative Taurocholate Uptake in Sandwich-Cultured Hepatocytes as Shown in Figure 4-5 ____

Model	A	B	C	D	E	F
1	K_a	K_{e1}	K_{e2}	-	-	-
2	K_{ma} V_{ma}	K_{e1}	K_{e2}	-	-	-
3	K_{ma} V_{ma}	K_{e1}	K_{me2} V_{me2}	-	-	-
4	K_{ma} V_{ma}	K_{e1}	K_{me2} V_{me2} K_{e2}	-	-	-
5	K_{ma} V_{ma}	K_{me1} V_{me1}	K_{me2} V_{me2}	-	-	-
6	K_a	-	-	K_{e3}	-	K_b
7	K_a	-	-	-	K_{e4}	K_b
8	K_a	-	-	K_{e3}	K_{e4}	K_b
9	K_{ma} V_{ma}	-	-	K_{e3}	-	K_b
10	K_{ma} V_{ma}	-	-	-	K_{e4}	K_b
11	K_{ma} V_{ma}	-	-	K_{e3}	K_{e4}	K_b
12	K_{ma} V_{ma}	-	-	K_{e3}	-	K_{mb} V_{mb}
13	K_{ma} V_{ma}	-	-	-	K_{e4}	K_{mb} V_{mb}
14	K_{ma} V_{ma}	-	-	K_{e3}	K_{e4}	K_{mb} V_{mb}
15	K_{ma} V_{ma} K_a	-	-	-	K_{e4}	K_{mb} V_{mb}
16	K_{ma} V_{ma}	-	-	-	K_{me4} V_{me4}	K_{mb} V_{mb}
17	K_{ma} V_{ma}	-	-	-	K_{e4}	K_{mb} V_{mb} K_b

Abbreviations: K_a , first-order rate constant for basolateral uptake; K_{e1} , first-order rate constant for elimination from cell and bile compartment in standard buffer; K_{e2} , first-order rate constant for elimination from cell and bile compartment in Ca^{2+} -free buffer; K_{ma} , Michaelis-Menten constant for basolateral uptake; V_{ma} , maximal velocity for basolateral uptake; K_{me2} , Michaelis-Menten constant for elimination from cell and bile compartment in Ca^{2+} -free buffer; V_{me2} , maximal velocity for elimination from cell and bile compartment in Ca^{2+} -free buffer; K_{me1} , Michaelis-Menten constant for elimination from cell and bile compartment in standard buffer; V_{me1} , maximal velocity for elimination from cell and bile compartment in standard buffer; K_{e3} , first-order rate constant for elimination from cell compartment in standard buffer; K_{e4} , first-order rate constant for elimination from bile compartment in standard buffer; K_b , first-order rate constant for biliary excretion; K_{mb} , Michaelis-Menten constant for biliary excretion; V_{mb} , maximal velocity for biliary excretion; K_{me4} , Michaelis-Menten constant for elimination from bile compartment in standard buffer; V_{me4} , maximal velocity for elimination from bile compartment in standard buffer; symbol '-', not applicable.

Table 4-2. Values of Akaike's Information Criterion (AIC) After Nonlinear Least-Squares Regression Analysis of Mean Cumulative Taurocholate Uptake Data Utilizing the Models Described in Figure 4-5 and Table 4-1

Model	AIC	Model	AIC
1	853.8	9	447.4
2	402.3	10	390.9
3	390.1	11	393.8
4	392.1	12	444.1
5	392.7	13	383.7
6	512.5	14	385.8
7	498.8	15	385.7
8	501.0	16	387.0
		17	385.8

Table 4-3. Kinetic Parameters^a Associated with Cumulative Uptake of Taurocholate in Hepatocytes Cultured in a Sandwich Configuration (n = 3)

Parameters	Mean \pm SE
K_{ma} (μ M)	28.0 ± 3.64
V_{ma} ($\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$)	1.19 ± 0.07
K_{e4} (min^{-1})	0.849 ± 0.074
K_{mb} ($\text{nmol} \cdot \text{mg protein}^{-1}$)	1.03 ± 0.35
V_{mb} ($\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$)	1.82 ± 0.36

^aParameters were obtained by fitting Model 13 to the data in Figure 4-6 with nonlinear least-squares regression analysis.

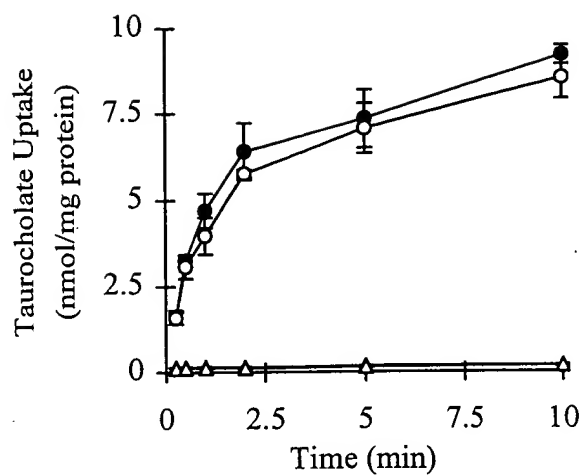


Figure 4-1. Cumulative uptake of [³H]taurocholate (25 μ M) in standard buffer (closed symbols) or Ca²⁺-free buffer (open symbols) in freshly isolated hepatocytes at 37°C (circles) or 4°C (triangles).

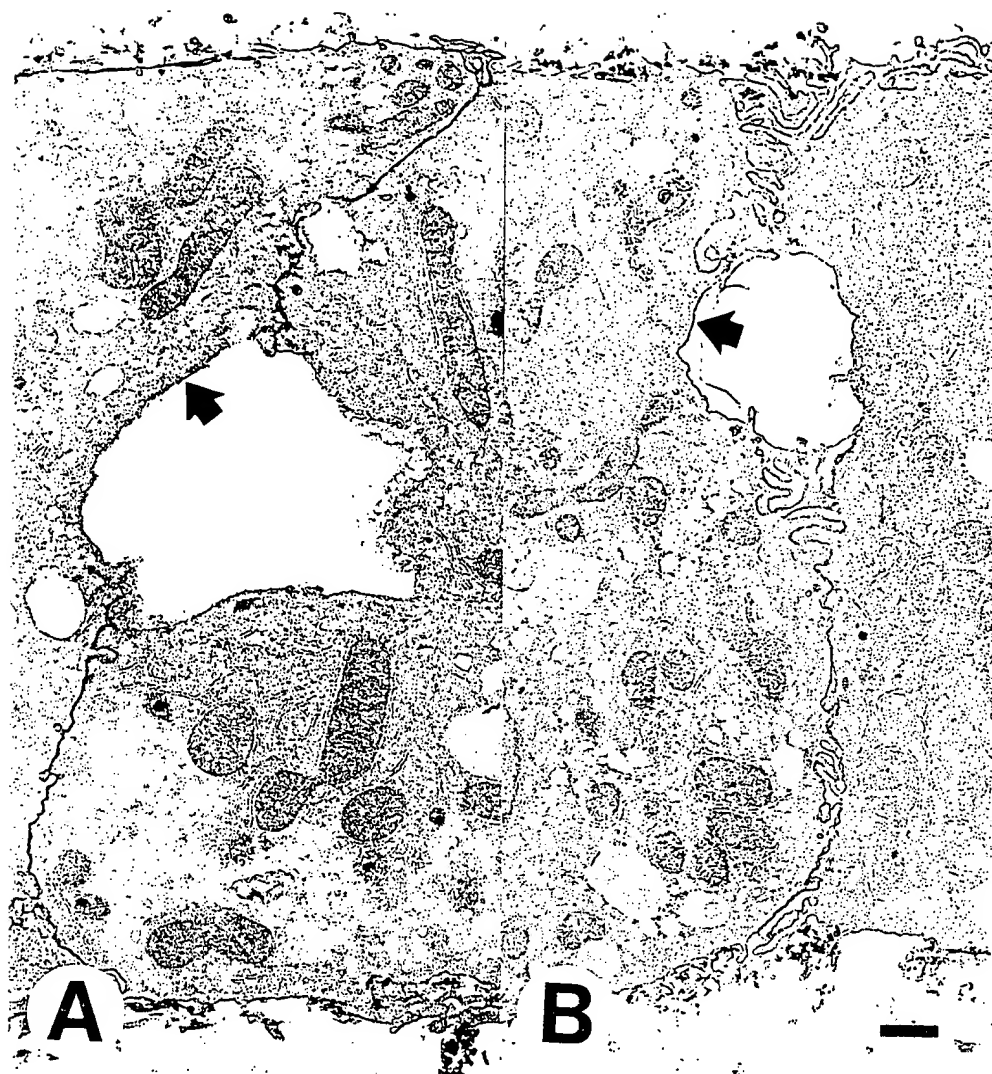


Figure 4-2. Effects of Ca^{2+} depletion on the integrity of tight junctions in hepatocytes cultured in a sandwich configuration for 4 days. In standard buffer (A) ruthenium red staining was present along intercellular membranes but not on the canalicular membranes (arrow). Tight junctions prevent ruthenium red from reaching the canalicular membrane surface. In Ca^{2+} -free buffer (B) ruthenium red staining was present along intercellular and canalicular membranes (arrow). Bar = 1 μm .

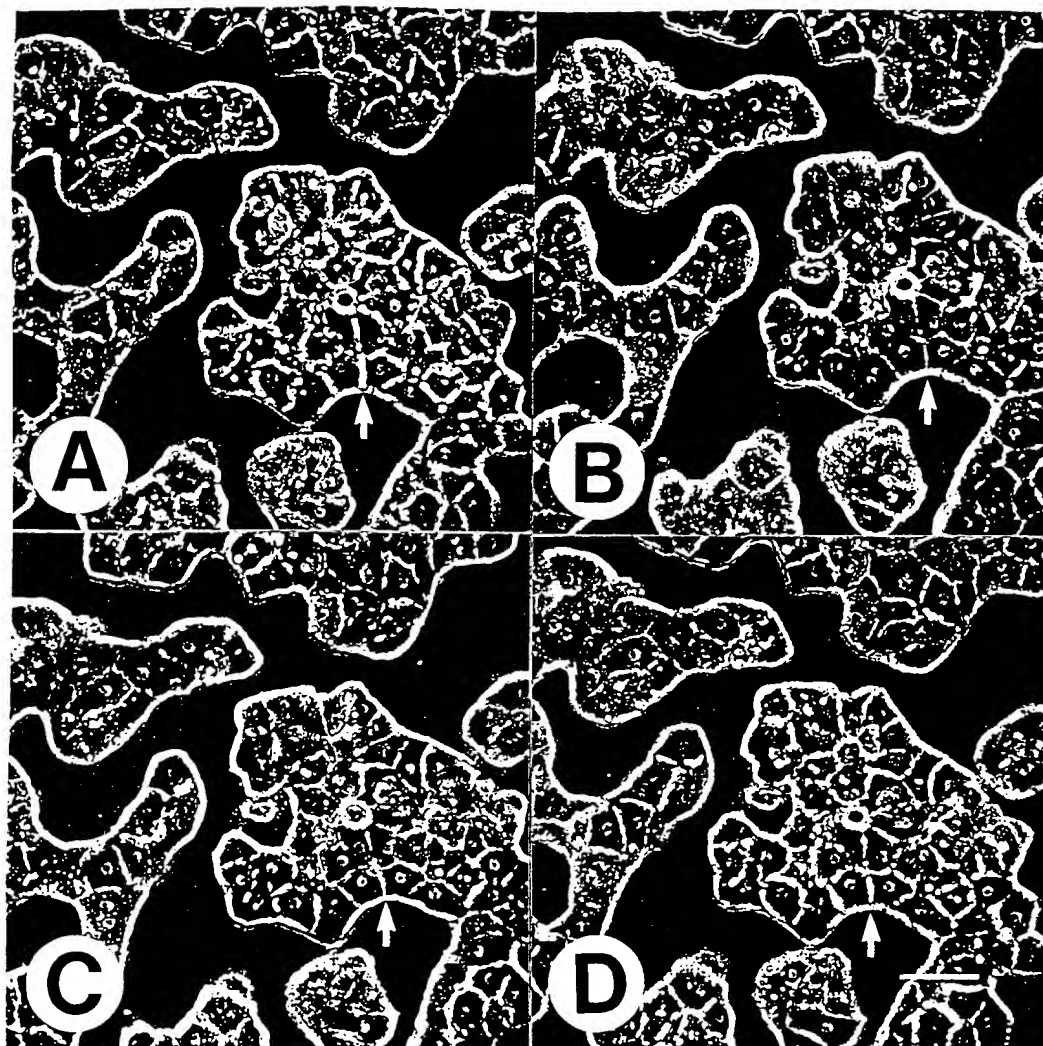


Figure 4-3. Effects of Ca^{2+} modulation on hepatocyte morphology in long-term sandwich-cultured hepatocytes. The monolayers cultured in a sandwich configuration for 4 days (A) were incubated in Ca^{2+} -free buffer for 10 min (B), followed by incubation in standard buffer for 10 min (C) and 60 min (D). After incubation of the monolayers for 10 min in Ca^{2+} -free buffer, the size of the canaliculi was reduced drastically (Figure 4-3B, arrow). Canaliculi size did not change considerably after incubation of the monolayers in standard buffer for 10 min (Figure 4-3C, arrow), but after incubation in standard buffer for 60 min, the majority of canaliculi had re-dilated (Figure 4-3D, arrow). Bar = 50 μm .

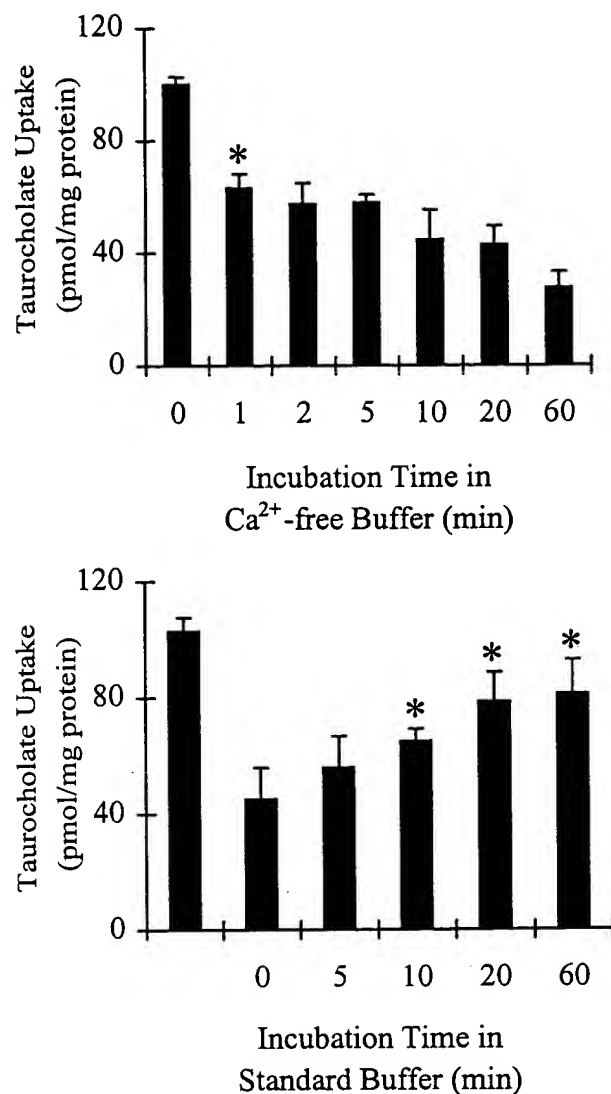


Figure 4-4. Cumulative uptake (10 min) of [^3H]taurocholate in hepatocytes cultured in a sandwich configuration for 4 days. Effects of Ca^{2+} depletion (A). After the monolayers were incubated in Ca^{2+} -free buffer for designated times, cumulative uptake was determined by incubation of the monolayers in standard buffer containing 1 μM [^3H]taurocholate for 10 min. * Uptake at designated times after incubation in Ca^{2+} -free buffer was statistically different ($p < 0.05$) from uptake after incubation in Ca^{2+} -free buffer for 10 min. Effects of incubation time in standard buffer (B). Hepatocyte monolayers were pre-incubated in Ca^{2+} -free buffer for 10 min prior to incubation in standard buffer at designated times. Cumulative uptake was determined by incubation of the monolayers in standard buffer containing 1 μM [^3H]taurocholate for 10 min. Control represents 10-min cumulative uptake in hepatocyte monolayers not pre-incubated in Ca^{2+} -free buffer. * Uptake at designated times after incubation in standard buffer was statistically different ($p < 0.05$) from time 0 (no incubation in standard buffer prior to initiation of 10-min cumulative uptake).

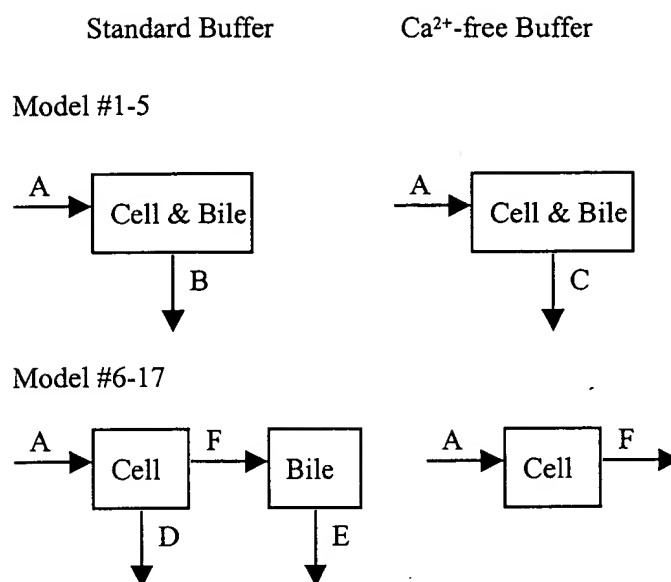


Figure 4-5. Model scheme describing the cumulative uptake of taurocholate in hepatocytes cultured in a sandwich configuration for 4 days. Parameter designations are provided in Table 4-1.

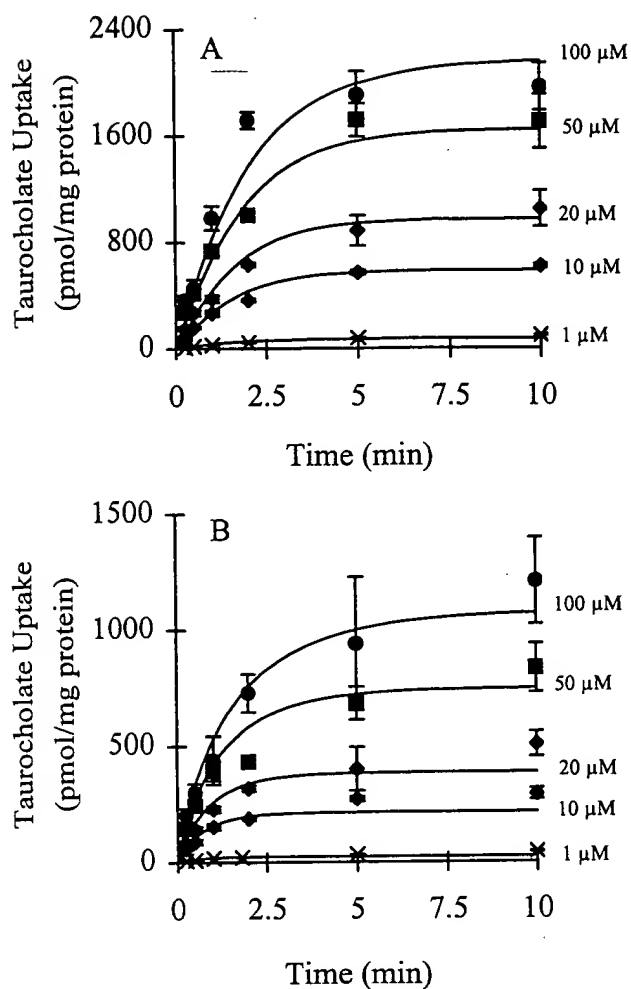


Figure 4-6. Cumulative uptake of taurocholate in hepatocytes cultured in a sandwich configuration for 4 days. The hepatocyte monolayers were pre-incubated in standard buffer (A) or Ca^{2+} -free buffer (B) for 10 min before the transport studies were initiated in standard buffer containing [^3H]taurocholate (1-100 μM). Lines represent the computer-generated best fit of the compartmental model equations (Model 13) to the data.

CHAPTER 5

CORRELATION OF BILIARY EXCRETION IN SANDWICH-CULTURED RAT HEPATOCYTES AND *IN VIVO* IN RATS

This chapter has been submitted to the *Drug Metabolism and Disposition*, and is presented in the style of that journal.

ABSTRACT

The relationship between biliary excretion in sandwich-cultured rat hepatocytes and biliary clearance *in vivo* in rats was examined. The biliary excretion of 7 model substrates in 96-hr sandwich-cultured rat hepatocytes was determined by differential cumulative uptake of substrate in monolayers pre-incubated in standard buffer (intact bile canaliculi) versus Ca^{2+} -free buffer (disrupted bile canaliculi). Biliary excretion *in vivo* was quantitated in bile duct cannulated rats. The Biliary Excretion Index of model substrates, equivalent to the percentage of retained substrate localized in the canalicular networks, was consistent with the percentage of the dose excreted in bile from *in vivo* experiments. The *in vitro* biliary clearance of inulin, salicylate, methotrexate, [D-Pen^{2,5}]enkephalin, and taurocholate, calculated as the ratio of the amount excreted into the bile canalicular networks to the area under the incubation-medium-concentration-time profile (~ 0 , ~ 0 , 4.1 ± 1.0 , 12.6 ± 2.2 , and 56.2 ± 6.0 ml/min/kg, respectively), correlated with the intrinsic *in vivo* biliary clearance of these compounds (0.04, 0, 17.3, 34.4, and 116.9 ml/min/kg, respectively; $r^2 = 0.9865$). The model compound 264W94 was not excreted in bile, either *in vivo* or *in vitro*. The glucuronide conjugate of 2169W94, the O-demethylated metabolite of 264W94, was excreted into bile *in vitro* when 2169W94, but not 264W94, was incubated with the monolayers; 2169W94 glucuronide undergoes extensive biliary excretion after administration of 264W94 or 2169W94 *in vivo*. The results from these studies show that biliary excretion in long-term sandwich-cultured rat hepatocytes correlates with *in vivo* biliary excretion. The application of this *in vitro* model system to study *in vivo* biliary excretion of metabolites requires consideration of the expression of individual isoforms of phase I and phase II enzymes in the monolayers.

INTRODUCTION

Many drugs undergo biliary excretion, although the extent of secretion in bile often is difficult to quantitate, particularly in humans. Alterations in biliary excretion due to disease states or drug interactions may have important pharmacologic and/or toxicologic implications. For example, co-administration of antibiotics with drugs that undergo enterohepatic recirculation may inhibit substantially enterohepatic recycling and result in lower plasma concentrations and pharmacologic effects of the affected drug (1). This type of drug interaction has been implicated in the failure of the oral contraceptive ethinylestradiol (2). Interactions at the level of biliary excretion are difficult to predict due to the lack of an optimal *in vitro* model to evaluate and study biliary excretion.

Elucidation of biliary excretion properties of drug candidates is a critical issue in the drug discovery and development process. Drug candidates that are excreted extensively into bile may never achieve adequate concentrations *in vivo*. For example, many metabolically stable peptides exhibit short residence times in the systemic circulation (3, 4) and low bioavailability after oral administration due to rapid and extensive biliary excretion (5, 6). Therefore, knowledge of the extent of biliary excretion of drug candidates in the early stages of drug development may be as important as absorption and metabolic properties when selecting drug candidates.

Numerous *in vivo* (e.g., bile duct cannulated animals) and *in vitro* preparations (e.g., isolated perfused livers, isolated hepatocytes, hepatocyte couplets, liver plasma membrane vesicles and expressed transport proteins) have been used to investigate biliary excretion processes (7). However, existing methods may not always be applied to investigations of

human biliary excretion. In addition, current approaches cannot be used to examine efficiently biliary excretion processes for a large number of drug candidates. Therefore, there is substantial need for a rapid and inexpensive *in vitro* screening method that is predictive of hepatobiliary disposition in animals and humans, especially, in this modern era of high synthetic capabilities (*i.e.*, combinatorial chemistry approaches).

Long-term (more than 24 hr) sandwich-cultured hepatocytes represent a potential *in vitro* model system to study biliary excretion. Previous work has demonstrated that maintenance of hepatocytes in a collagen-sandwich configuration prolongs cell viability and preserves liver-specific protein synthesis (8, 9). Further studies showed that long-term sandwich-cultured hepatocytes reestablish a structurally and functionally normal bile canalicular network and maintain drug uptake and enzyme-induction potential (10, 11, 12). Recently, it has been demonstrated that the expression and function of primary active transporters, such as the sinusoidal Na^+ /taurocholate cotransporting polypeptide, the canalicular bile acid transporter, and the canalicular multispecific organic anion transporter, were maintained in hepatocytes cultured in a collagen-sandwich configuration for 96-120 hr (Chapter 2, Chapter 3).

The sandwich-cultured hepatocyte system is composed of two compartments: cytosol and canalicular lumen. The tight junctional complex is the diffusional barrier between the canalicular lumen and the extracellular space (13, 14). In this system, Ca^{2+} depletion increases tight junction permeability and enables substrate translocation between the canalicular and extracellular spaces based on concentration gradients (Chapter 3). During cumulative uptake studies, substrate in the medium was taken up by hepatocytes, and

excreted into the bile canalicular networks. In standard buffer, the barrier function of the tight junctions is intact, and the excreted substrate is localized in the canalicular compartment. In Ca^{2+} -free buffer, the barrier function of tight junctions is disrupted, and the substrate in the canalicular compartment diffuses back into the incubation medium. Thus, in standard buffer, the cumulative uptake of a substrate in the long-term sandwich cultured hepatocytes represents the amount of substrate in the cytosolic and canalicular compartments; in Ca^{2+} -free buffer, the cumulative uptake represents substrate in the cytosolic compartment. The amount of substrate secreted in the canalicular lumen, the biliary excretion of substrates in the monolayers, can be estimated from the difference in cumulative uptake in the presence and absence of Ca^{2+} . This method has been utilized to study quantitatively the formation of hepatocyte polarization during the course of culture (Chapter 3). However, it remains to be determined whether estimates of biliary excretion based on this *in vitro* model are consistent with *in vivo* biliary excretion data.

The objective of the present study was to examine the relationship between the estimated biliary excretion in the long-term sandwich-cultured hepatocytes and the extent of biliary excretion *in vivo* in rats. These results indicate that biliary excretion in rat hepatocytes cultured in a collagen-sandwich configuration for 96 hr correlates with *in vivo* biliary excretion in rats.

MATERIALS AND METHODS

Chemicals. [^3H]Taurocholate (3.4 Ci/mmol; purity > 97%), [^{14}C]salicylate (55.5 mCi/mmol; purity > 99%), and [^3H][D-Pen 2,5]enkephalin (36 Ci/mmol; purity > 97%) were obtained from Dupont New England Nuclear (Boston, MA). [^3H]Methotrexate (13.7 Ci/mmol; purity > 99%) and [^3H]inulin (1.3 Ci/mmol; purity > 97%) were from Amersham International plc (Buckinghamshire, England). Compounds [^{14}C]264W94 ((3R, 5R)-3-butyl-3-ethyl-2, 3, 4, 5-tetrahydro-7, 8-dimethoxy-5-phenyl-1, 4-benzothiazepine-1, 1-dioxide; 45.5 mCi/mmol; purity > 99%) and [^{14}C]2169W94 ((3R, 5R)-3-butyl-3-ethyl-2, 3, 4, 5-tetrahydro-7-methoxy-8-hydroxy-5-phenyl-1, 4-benzothiazepine-1, 1-dioxide; 43.7 mCi/mmol; purity > 99%) were obtained from Glaxo Wellcome, Inc. (Research Triangle Park, NC). Collagenase (type I, class I) was obtained from Worthington Biochemical Corp. (Freehold, NJ). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum and insulin were purchased from Gibco (Grand Island, NY). Rat tail collagen (type I) was obtained from Collaborative Biomedical Research (Bedford, MA). All other chemicals and reagents were of analytical grade and were readily available from commercial sources.

Animals. Male Wistar rats (250-280 g) from Charles River Laboratory (Raleigh, NC) were used as liver donors. Rats were housed individually in stainless-steel cages in a constant alternating 12-hr light and dark cycle at least 1 week before the study was performed, and were fed *ad libitum* until use. Bile duct cannulated rats (200-250g) were obtained from Charles River (Raleigh, NC). All procedures were approved by the Institutional Animal Care and Use Committee.

Preparation of Culture Dishes. Plastic culture dishes (60 mm) were precoated with rat tail collagen at least 1 day prior to preparing the hepatocyte cultures. To obtain a gelled collagen substratum, ice-cold neutralized collagen solution (0.1 ml, 1.5 mg/ml, pH 7.4) was spread onto each culture dish. Freshly coated dishes were placed at 37°C in a humidified incubator for approximately 1 hr to allow the matrix material to gel, followed by addition of 3 ml DMEM to each dish and storage in a humidified incubator.

Culture of Rat Hepatocytes. Hepatocytes were isolated with a two-step perfusion method as reported previously (Chapter 2). Rats were anesthetized with ketamine and xylazine (60 and 12 mg/kg i.p., respectively) prior to portal vein cannulation. The liver was perfused *in situ* with oxygenated Ca^{2+} -free Krebs-Henseleit bicarbonate buffer containing 5.5 mM glucose for 10 min at 37°C followed by perfusion with Krebs-Henseleit bicarbonate buffer containing collagenase type I (0.5 mg/ml) for 10 min. The hepatic capsule was removed with forceps. The hepatocytes were released by shaking the liver gently in 100 ml DMEM. The released cells were filtered through a sterile nylon mesh (70- μm). The hepatocyte suspensions were centrifuged at 50 \times g for 3 min. The cell pellet was resuspended in 25 ml DMEM and an equal volume of 90% isotonic Percoll (pH 7.4); the resulting cell suspension was centrifuged at 150 \times g for 5 min. The pellet was resuspended in 50 ml DMEM and the cell suspensions were combined into one tube followed by centrifugation at 50 \times g for 3 min. Hepatocyte viability was determined by trypan blue exclusion. Only those hepatocyte preparations with viability greater than 90% were utilized for further studies. Hepatocyte suspensions were prepared with DMEM containing 5% fetal calf serum, 1 μM dexamethasone and 4 mg/L insulin. Hepatocyte suspensions were added to the pre-coated

dishes at a density of 2×10^6 cells/60-mm dish. Approximately 1 hr after plating the cells, the medium was aspirated and 3-ml fresh DMEM was added. For transport studies, hepatocytes that had been seeded for 3-5 hr without collagen overlay were defined as 3-hr or short-term cultured hepatocytes.

To prepare sandwich-cultured hepatocytes, neutralized collagen solution (0.1 ml, 1.5 mg/ml, pH 7.4) was added to the monolayers 24 hr after the cells were seeded. Cultures with collagen overlay were incubated for 45 min at 37°C in a humidified incubator to allow the collagen to gel before addition of DMEM. Medium was changed on a daily basis until the fourth day after the cells were seeded. These hepatocytes were referred to as 96-hr or long-term cultured hepatocytes.

Cumulative Uptake Studies in Sandwich-Cultured Hepatocytes. Hepatocytes cultured in a collagen-sandwich configuration were incubated in 3 ml standard buffer or Ca^{2+} -free buffer at 37°C for 10 min. After removing the incubation buffer, uptake was initiated by addition of 3 ml standard buffer containing substrate to each dish. After incubation for designated times, cumulative uptake was terminated by aspirating the incubation solution and rinsing 4 times with 3 ml ice-cold standard buffer to remove extracellular substrate. After washing, 2 ml of 1% Triton X-100 solution was added to culture dishes, and the cells were lysed by shaking the dish on a shaker for 20 min at room temperature. An aliquot (1 ml) of lysate was analyzed by liquid scintillation spectrometry. Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA) was used to determine the protein concentration in the culture extracts using bovine serum albumin as standard. Triton X-100 (1%) did not interfere with the assay. All values for substrate uptake into cell

monolayers were corrected for nonspecific binding to the collagen by subtracting the substrate uptake determined in the appropriate control dishes in the absence of cells as described previously.

Biliary Excretion in Rats after Intravenous Administration of 264W94 and Oral Administration of 2169W94. [^{14}C]264W94 was formulated as a solution in a mixture of sterile water/polypropylene glycol 400/ethanol (2:1:1 v/v/v) at a concentration of 0.125 mg/ml. Following collection of pre-dose bile, [^{14}C]264W94 solution was administered by caudal vein injection (0.1 mg/kg). For the 2169W94 studies, [^{14}C]2169W94 was prepared as a suspension at a concentration of 0.1 mg/ml in 0.5% (w/v) methylcellulose in water. Following collection of pre-dose bile, [^{14}C]2169W94 suspension was administered by gavage (1.0 mg/kg). All rats were placed in individual plastic metabolism cages that allowed the rats unrestrained movement. Bile was collected in polypropylene containers surrounded by ice. For the 264W94 studies, the bile container was changed at 1, 2, 3, 4, 5, 6, 12, and 24 hours after the dose; for the 2169W94 studies, the container was changed at 8 and 24 hours after the dose. Previous studies indicated that samples were stable on ice for 24 hours. Bile samples were stored at -20°C until analysis.

Analytical Procedure. Aliquots of cell lysate or bile samples containing 264W94 or 2169W94 were mixed with 2 volumes of ice-chilled acetonitrile, and centrifuged to remove precipitated proteins. The supernatant was evaporated under nitrogen at room temperature, and reconstituted in 100 μL of a 70/30 mixture of 50 mM ammonium acetate/acetonitrile/trifluoroacetic acid (95:5:0.1 v:v:v) and acetonitrile. The sample extracts were injected onto a WatersTM Symmetry C18 column (3.9 x 150 mm) and eluted with a

85/15 mixture of 50 mM ammonium acetate (pH 4.0) and acetonitrile; the percentage of acetonitrile was increased by a Waters 600E System Controller to 55% over a period of 20 minutes, and then to 100% during the next 10 minutes. Radiocarbon that eluted from the HPLC was quantitated with an on-line radioactivity detector (Radiomatic Flo-One/Beta Radio-Chromatography Detector Series 500TR Series, Packard Instrument Co.). The peaks associated with 264W94, 2169W94, and 2169W94 glucuronide were identified by comparing with purified standard compound. Under these conditions, baseline separation of these three components was achieved. The concentration of the three components was determined by normalizing the eluted radioactivity in each peak to the total injected radioactivity.

Data Analysis. Uptake data were normalized to the protein content and expressed as mean \pm SD from 3-4 separate preparations of hepatocytes. Statistical differences between mean values for the 10-min cumulative substrate uptake in the presence and absence of Ca^{2+} were determined by Student's *t*-test. A *P* value of < 0.05 was considered significant.

In vivo biliary clearance, Cl_B (ml/min/kg body weight), was calculated according to Equation 1:

$$\text{Cl}_B = \frac{\text{Amount}_{\text{bile}(0-T)}}{\text{AUC}_{0-T}} \quad \text{Equation 1}$$

where $\text{Amount}_{\text{bile}(0-T)}$ represents the amount of parent drug recovered in bile from time 0 to time T, and AUC_{0-T} represents the area under the plasma concentration-time curve from 0 to time T.

The *in vivo* intrinsic biliary clearance (Cl_{Bin} , ml/min/kg body weight) was estimated according to Equation 2 based on the well-stirred model of hepatic disposition assuming biliary excretion is the predominant elimination pathway (15).

$$Cl_{Bin} = \frac{Q \cdot Cl_B}{Q - Cl_B} \quad \text{Equation 2}$$

where Q represents rat hepatic plasma flow, 40 ml/min/kg of body weight (blood flow \times hematocrit; 16), and Cl_B represents biliary clearance for model compounds reported in the literature or calculated from Equation 1.

Biliary excretion of substrates in the monolayers was assessed quantitatively by the Biliary Excretion Index (Chapter 3) based on Equation 3:

$$\text{Biliary Excretion Index} = \frac{\text{Uptake}_{\text{standard}} - \text{Uptake}_{\text{Ca}^{2+}\text{-free}}}{\text{Uptake}_{\text{standard}}} \cdot 100\% \quad \text{Equation 3}$$

where $\text{Uptake}_{\text{standard}}$ and $\text{Uptake}_{\text{Ca}^{2+}\text{-free}}$ represent the cumulative uptake of substrate over a 10-min interval in the hepatocyte monolayers pre-incubated in standard buffer and in Ca^{2+} -free buffer, respectively.

Biliary clearance in the sandwich-cultured hepatocytes, $Cl_{B(\text{culture})}$ (ml/min/kg per body weight), was calculated according to Equation 4:

$$Cl_{B(\text{culture})} = \frac{\text{Uptake}_{\text{standard}} - \text{Uptake}_{\text{Ca}^{2+}\text{-free}}}{\text{Time}_{\text{incubation}} \cdot \text{Concentration}_{\text{medium}}} \quad \text{Equation 4}$$

where $\text{Time}_{\text{incubation}}$ was 10 min and $\text{Concentration}_{\text{medium}}$ represents the initial substrate concentration in the incubation medium. Rat liver weight and protein content in liver tissue were assumed to be 40 g/kg of body weight and 0.20 g/g of liver weight (17), respectively, in all calculations.

RESULTS

Cumulative Uptake in Cultured Hepatocytes.

The cumulative uptake of inulin was negligible (less than 0.01% of initial added substrate) at all incubation times in either short-term or long-term cultured hepatocytes (Figure 1A and B). In the 3-hr cultured hepatocytes, the cumulative uptake of salicylate, methotrexate and [D-Pen^{2,5}]enkephalin was not significantly different in standard buffer and in Ca²⁺-free buffer (2A, 3A, and 4A; $p > 0.05$). However, slightly higher cumulative uptake of taurocholate in standard buffer compared to Ca²⁺-free buffer was observed (Figure 5A); at 10 min, the cumulative uptake in standard buffer was approximately 10% higher than in Ca²⁺-free buffer ($p = 0.0352$). In 96-hr cultured hepatocytes, extracellular Ca²⁺ had no effect on the cumulative uptake of salicylate (Figure 2B, $p > 0.05$). However, the uptake of methotrexate, [D-Pen^{2,5}]enkephalin, and taurocholate in long-term cultured hepatocytes in standard buffer was significantly higher than in Ca²⁺-free buffer (Figure 3B, 4B, and 5B; $p < 0.05$).

Relationship Between the Percentage of Dose Excreted in Bile in Rats and Biliary Excretion Index in Cultured Hepatocytes.

Five model substrates representing a diverse spectrum of biliary excretion properties were selected to examine the relationship between the percentage of the dose excreted in bile *in vivo* in rats and the Biliary Excretion Index in sandwich-cultured hepatocytes. Information regarding the percentage of the dose excreted in rat bile after i.v. administration was obtained from the literature. The extent of inulin and salicylate secretion into bile was negligible (18, 19). Approximately 50-60% of a 22- μ mol/kg methotrexate dose (20, 21) and 70% of a 14.5-

$\mu\text{mol/kg}$ [D-Pen^{2,5}]enkephalin dose (4) were excreted into rat bile as unchanged drug in 1 hr. Taurocholate biliary excretion was—more rapid and extensive than biliary excretion of methotrexate and [D-Pen^{2,5}]enkephalin. In 1 hr, virtually 100% of the dose ($8.0 \mu\text{mol/kg}$) was recovered in rat bile (22).

Biliary excretion in the sandwich-cultured hepatocytes can be expressed quantitatively as the Biliary Excretion Index calculated from Equation 3 based on the 10-min cumulative uptake data in Figure 3B-5B. The Biliary Excretion Index of inulin and salicylate was assumed to be negligible because no statistically significant differences in the cumulative uptake of inulin or salicylate were observed between standard buffer and Ca^{2+} -free buffer ($p > 0.05$). The Biliary Excretion Index of methotrexate, [D-Pen^{2,5}]enkephalin and taurocholate was $55.4 \pm 18.3\%$, $42.4 \pm 6.5\%$, and $56.4 \pm 5.2\%$, respectively. The relationship between the percentage of the dose excreted in rat bile *in vivo* and the Biliary Excretion Index measured in the *in vitro* system is depicted in Figure 6A. The Biliary Excretion Index was very low for compounds undergoing negligible biliary excretion *in vivo* (*i.e.*, inulin and salicylate). In contrast, the Biliary Excretion Index was moderately high for compounds that are excreted in bile *in vivo* (*i.e.*, methotrexate, [D-Pen^{2,5}]enkephalin, and taurocholate).

Correlation of *In Vitro* and *In Vivo* Biliary Clearance.

The *in vivo* biliary clearance (ml/min per kg body weight) of inulin, salicylate, methotrexate, and taurocholate was 0.035 (18), ~0 (19), 12.1 (21), and 29.8 (22), respectively. *In vivo* biliary clearance of [D-Pen^{2,5}]enkephalin, 18.5 ml/min/kg, was calculated based on Equation 1 from the data reported by Chen and Pollack (4). Based on these *in vivo* biliary clearance values, the intrinsic biliary clearance of inulin, salicylate,

methotrexate, [D-Pen^{2,5}]enkephalin, and taurocholate was calculated from Equation 2 (0.04, 0, 17.3, 34.4, and 116.9 ml/min/kg, respectively). The *in vitro* biliary clearance of inulin, salicylate, methotrexate, [D-Pen^{2,5}]enkephalin, and taurocholate, calculated from Equation 4 based on the 10-min cumulative uptake data (Figures 1B-5B), was ~0, ~0, 4.1 ± 1.0 , 12.6 ± 2.2 , and 56.2 ± 6.0 ml/min/kg, respectively. The *in vivo* intrinsic biliary clearance correlated well with the *in vitro* biliary clearance ($r^2 = 0.9865$) for the five model compounds (Figure 6B).

Comparison of *In Vivo* and *In Vitro* Biliary Excretion of 264W94 and its Metabolites.

The structures of compounds 264W94 and 2169W94 are presented in Figure 7. Compound 2169W94 is the O-demethylated metabolite of 264W94 in rats and humans, which can undergo further conjugation with uridine-5'-diphosphoglucuronic acid to form a glucuronide conjugate (23).

After i.v. administration of [¹⁴C]264W94 to rats (0.24 μ mol/kg), neither 264W94 nor 2169W94 was detected in bile in 24 hr. However, 35.4% (n=2) of the total administered radioactivity was recovered in bile in the first hour. Approximately 30% of the radioactivity recovered in bile was the 2169W94 glucuronide; the remaining 70% of radioactivity in bile represented unidentified metabolites. After oral administration of [¹⁴C]2169W94 to rats (2.4 μ mol/kg), 2169W94 was not detected in the bile in 24 hr. However, 66.4% (n=2) of the total administered radioactivity was recovered in bile in 8 hr. Approximately 88.7% of the radioactivity in bile was in the form of the 2169W94 glucuronide conjugate. These *in vivo* results demonstrate that 264W94 and its O-demethylated product, 2169W94, undergo

negligible biliary excretion, but the glucuronide conjugate of 2169W94 undergoes extensive biliary excretion in rats.

To determine the biliary excretion of 264W94 and metabolites in 3-hr and 96-hr cultured hepatocytes, hepatocyte monolayers were incubated in standard or Ca^{2+} -free buffer before cumulative uptake was conducted in standard buffer containing 3 μM of [^{14}C]264W94 or [^{14}C]2169W94 (Figure 8 and 9). In 3-hr cultured hepatocytes, the cumulative uptake measured by total radioactivity of 264W94 or 2169W94 was similar in the hepatocytes pre-incubated in standard buffer or Ca^{2+} -free buffer ($p > 0.05$), suggesting that the uptake of 264W94 and 2169W94 in short-term cultured hepatocytes was not affected by pre-incubation of the monolayers in Ca^{2+} -free buffer. In 96-hr cultured hepatocytes, the 10-min cumulative uptake of 264W94 measured by total radioactivity was not significantly different in the monolayers pre-incubated in standard buffer or Ca^{2+} -free buffer ($p > 0.05$). HPLC analysis of the cell lysate at 10 min revealed that 73.0% of the total radioactivity was in the form of 264W94 and 3.3% was the 2169W94 glucuronide conjugate; 2169W94 was not detected in the lysate. In 96-hr sandwich-cultured hepatocytes, 10-min cumulative uptake of 2169W94 was approximately 70% higher in the presence of Ca^{2+} than in the absence of Ca^{2+} ($p < 0.05$). In the 10-min cell lysate, approximately 16.7% of total radioactivity was in the form of 2169W94, and approximately 58.5% was the 2169W94 glucuronide conjugate. Compound 2169W94 forms the glucuronide conjugate which is excreted into bile canaliculi networks in long-term cultured hepatocytes.

DISCUSSION

Previous studies have indicated that long-term primary rat hepatocytes cultured between two layers of gelled collagen (sandwich configuration) maintain normal morphology, form extensive canalicular networks, and sustain many liver-specific functions (8, 9, 12). Recently, it has been demonstrated that the biliary excretion of the non-fluorescent substrate, taurocholate, in sandwich-cultured hepatocytes can be estimated as the difference in cumulative uptake of taurocholate in monolayers pre-incubated in standard buffer and in Ca^{2+} -free buffer (Chapter 3).

In contrast to long-term cultured hepatocytes, intact bile canaliculi are not prominent in short-term cultured hepatocytes (13, 14). Therefore, the cumulative uptake of compounds in short-term cultured hepatocytes only revealed the effects of Ca^{2+} on the membrane transport properties. The cumulative uptake of inulin, salicylate, methotrexate, and [D-Pen^{2,5}]enkephalin did not differ in the short-term cultured hepatocytes, suggesting that extracellular Ca^{2+} modulation had no effect on the membrane transport properties of these model substrates. Interestingly, the cumulative uptake of taurocholate in short-term cultured hepatocytes was slightly higher in standard buffer than in Ca^{2+} -free buffer. This difference may be related to the existence of hepatocyte couplets in the short-term cultures (24).

Biliary excretion of the 5 model substrates in long-term cultured hepatocytes was consistent with the *in vivo* biliary excretion of these compounds. Quantitation of biliary excretion in the cultured hepatocytes utilizing the Biliary Excretion Index has been described previously (Chapter 3). The Biliary Excretion Index represents the percentage of retained substrate in the bile canaliculi. Results indicate that compounds undergoing negligible

biliary excretion *in vivo* based on the percentage of the dose excreted in bile (*e.g.*, inulin, salicylate) have a low Biliary Excretion Index (~ 0), and compounds that are more extensively excreted in bile *in vivo* (*e.g.* methotrexate, [D-Pen^{2,5}]enkephalin, and taurocholate) have a high Biliary Excretion Index ($\sim 50\%$).

The relationship between the Biliary Excretion Index and the percentage of the dose excreted in bile *in vivo* reveals only a categorical correlation. Methotrexate and [D-Pen^{2,5}]enkephalin represent compounds that are “highly” excreted in bile (approximately 60% and 70% of the i.v. dose was recovered in bile in 1 hr, respectively). In contrast, taurocholate is “rapidly and extensively” excreted in biliary (almost all of the i.v. dose excreted in bile in less than 1 hr). The Biliary Excretion Index can differentiate between compounds that undergo extensive versus negligible or low biliary biliary excretion. However, the Biliary Excretion Index does not appear to be able to differentiate between compounds that are highly excreted in bile, like methotrexate (Biliary Excretion Index: $\sim 55\%$) or [D-Pen^{2,5}]enkephalin (Biliary Excretion Index: $\sim 42\%$), and compounds that are rapidly and extensively excreted in bile, like taurocholate (Biliary Excretion Index: $\sim 56\%$). This limitation in the Biliary Excretion Index may be due to the fact that this index is determined predominantly by the canalicular excretory function; the percentage of the i.v. administered substrate excreted into bile *in vivo* is determined by sinusoidal uptake activity, canalicular excretory activity, and other competitive elimination processes.

Biliary clearance may represent a more appropriate parameter for comparison of the relationship between *in vivo* and *in vitro* biliary excretion. The *in vivo* biliary clearance was calculated in the present study as the ratio of the amount excreted into bile at time T and the

plasma AUC between time 0 and time T. Because most of the administered dose was eliminated at time T, the biliary clearance should approximate the biliary clearance calculated from time 0 to infinity. Biliary clearance calculated in this manner is a function of intrinsic biliary clearance and the hepatic plasma flow rate. To eliminate the effects of plasma flow, the intrinsic biliary clearance was calculated based on the well-stirred model of hepatic disposition (15). Likewise, *in vitro* biliary clearance was calculated as the ratio of the amount excreted into the canalicular networks in the hepatocyte monolayers and the AUC in the incubation medium. In the sandwich-cultured hepatocytes, the incubation medium was accessible to all hepatocytes on the dish at the same time. Thus, the calculated *in vitro* biliary clearance should represent the intrinsic biliary clearance. However, since biliary excretion involves two processes, uptake across the sinusoidal membrane and excretion across the canalicular membrane, the true intrinsic biliary clearance should be determined by transport across the canalicular membrane and calculated based on intracellular substrate concentrations. Therefore, the *in vivo* and *in vitro* “intrinsic” clearance values calculated in this study should be interpreted as “apparent” intrinsic biliary clearance values, which would be rate-limited by the slowest step in the process, either sinusoidal uptake or canalicular excretion.

The correlation between *in vitro* biliary clearance and *in vivo* intrinsic biliary clearance was high ($r^2 = 0.9865$) for the five model substrates. According to the *in vivo* intrinsic biliary clearance, the five model substrates can be classified into three groups: compounds that are not excreted in bile (inulin and salicylate; ~ 0 ml/min/kg), compounds that are excreted substantially in bile (methotrexate and [D-Pen^{2,5}]enkephalin; ~ 17.3 and

~34.4 ml/min/kg, respectively), and compounds that are rapidly and extensively excreted in bile (taurocholate; ~116.9 ml/min/kg). The estimated *in vitro* biliary clearance adequately differentiated between these three groups of compounds (~0, 4-13, and 56 ml/min/kg, respectively). These results suggest that the biliary clearance describes more accurately the relationship between *in vivo* and *in vitro* biliary excretion than the Biliary Excretion Index.

To further assess the utility of this *in vitro* model system to predict *in vivo* biliary excretion of drug metabolites, the *in vitro* and *in vivo* biliary excretion of 264W94, its O-demethylated metabolite (2169W94), and 2169W94 glucuronide were examined. *In vitro* studies conducted with rat and human liver microsomes, precision-cut liver slices, and cDNA expressed hepatic cytochrome P450 isozymes indicated that 264W94 formed an O-demethylated metabolite at the 8-methoxy position. Among several cytochrome P450 isozymes examined, CYP3A played a major role in the metabolism of 264W94 (23). *In vivo* disposition studies demonstrated that neither 264W94 nor its O-demethylated metabolite, 2169W94, was excreted in bile, but the 2169W94 glucuronide conjugate along with other unidentified metabolites were excreted extensively in bile. The lack of biliary excretion of 264W94 in long-term sandwich-cultured hepatocytes was consistent with negligible *in vivo* biliary excretion of 264W94. *In vivo*, approximately 35% of 264W94 equivalent was excreted in bile as metabolites in 1 hr after i.v. administration of 264W94. In cultured hepatocytes, however, the biliary excretion of 264W94 metabolites was negligible (Figure 8B). This apparent discrepancy between the *in vivo* and *in vitro* biliary excretion for the metabolites of 264W94 may be explained by differences in P450 enzyme levels. *In vivo*, 264W94 undergoes O-demethylation to form 2169W94; subsequently, 2169W94 is

conjugated with uridine-5'-diphosphoglucuronic acid to form 2169W94 glucuronide. This glucuronide conjugate accounts for 30% of the total amount excreted in the bile. In the lysate of long-term cultured hepatocytes, only ~3% of the total amount incubated was detected as the 2169W94 glucuronide conjugate. These results indicate that the long-term cultured hepatocytes were not capable of the O-demethylation reaction, which is consistent with the loss of 3A levels in cultured hepatocytes (25). Consequently, negligible glucuronide conjugate was formed and excreted in bile. However, after incubation of the monolayers with 2169W94, the O-demethylated metabolite of 264W94, 58.5% of 2169W94 was converted to glucuronide conjugates and significant biliary excretion was observed in the cultured hepatocytes (Figure 9B). Evidently, phase I metabolic activities such as O-demethylation deteriorate significantly, while the phase II metabolic activities such as glucuronide conjugation are maintained, at least in part, in the long-term sandwich-cultured hepatocytes. This observation was consistent with previous studies indicating that activities of phase II enzymes are preserved in cultured rat hepatocytes to a greater extent than those of phase I enzymes, such as CYP3A (12, 26, 27). The present studies indicate that sandwich-cultured hepatocytes can be employed to predict *in vivo* biliary excretion of a substrate in its parent form. The application of this *in vitro* model system to study and to predict *in vivo* biliary excretion of metabolites requires consideration of the expression of individual isoforms of phase I and phase II enzymes in the monolayers.

The uncertainty in predicting the biliary excretion of drug metabolites should not limit the utility of this *in vitro* model as a screening tool for predicting the biliary excretion of drug candidates *in vivo*. This *in vitro* model may provide adequate information regarding the

biliary clearance of a drug candidate in its parent form. The deterioration of phase I metabolic activity with maintenance of biliary transport may represent an advantage of this *in vitro* model system to differentiate the biliary excretion of parent drug versus metabolites.

Previous studies indicated that the extent to which individual P450 enzymes are expressed in cultured hepatocytes depends greatly on the medium and matrix conditions (12, 28, 29, 30). In order to predict the biliary excretion of metabolites, culture conditions will need to be optimized to maintain both hepatic transport as well as phase I and phase II enzyme activities. In addition, it was reported recently, that extensive bile canalicular networks form in sandwich-cultured human hepatocytes (31). Whether biliary excretion in cultured human hepatocytes correlates with biliary excretion *in vivo* in humans is the subject of ongoing investigations.

In summary, results of the present study suggest that biliary excretion measured by the Biliary Excretion Index and apparent biliary clearance in sandwich-cultured rat hepatocytes correlate with *in vivo* biliary excretion in rats. Biliary clearance represents a useful indicator of *in vivo* biliary excretion. Application of this *in vitro* model to study *in vivo* biliary excretion of drug metabolites is possible if the relevant metabolic activities in the *in vitro* model are maintained.

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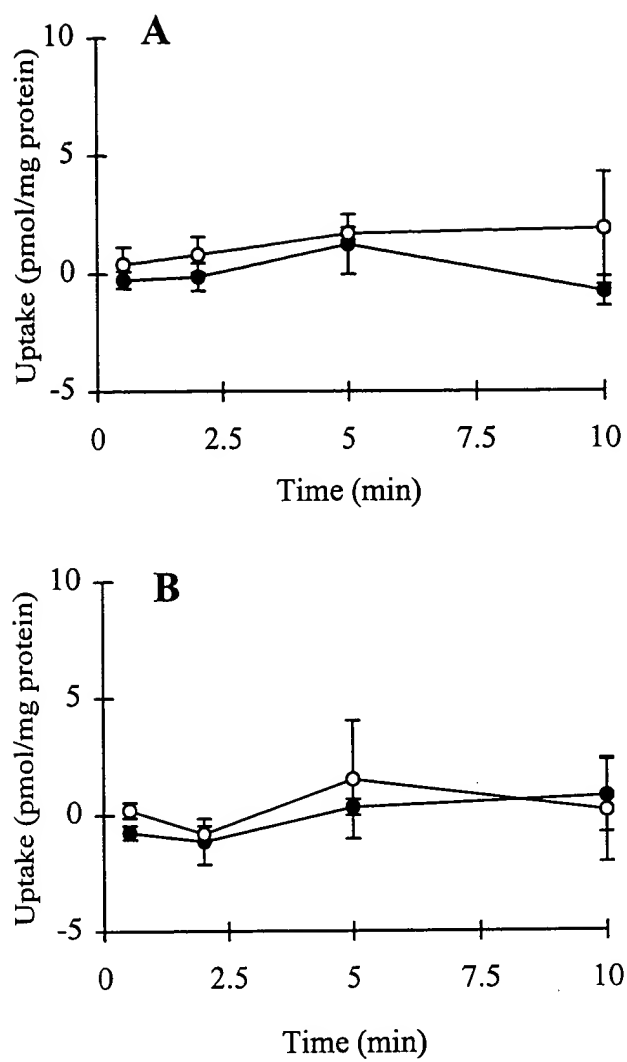


Figure 5-1. Cumulative uptake of $[^3\text{H}]$ inulin (1 μM) in standard buffer (closed symbols) and Ca^{2+} -free buffer (open symbols) in hepatocyte monolayers cultured for 3 hr (A) and hepatocytes cultured in a sandwich configuration for 96 hr (B).

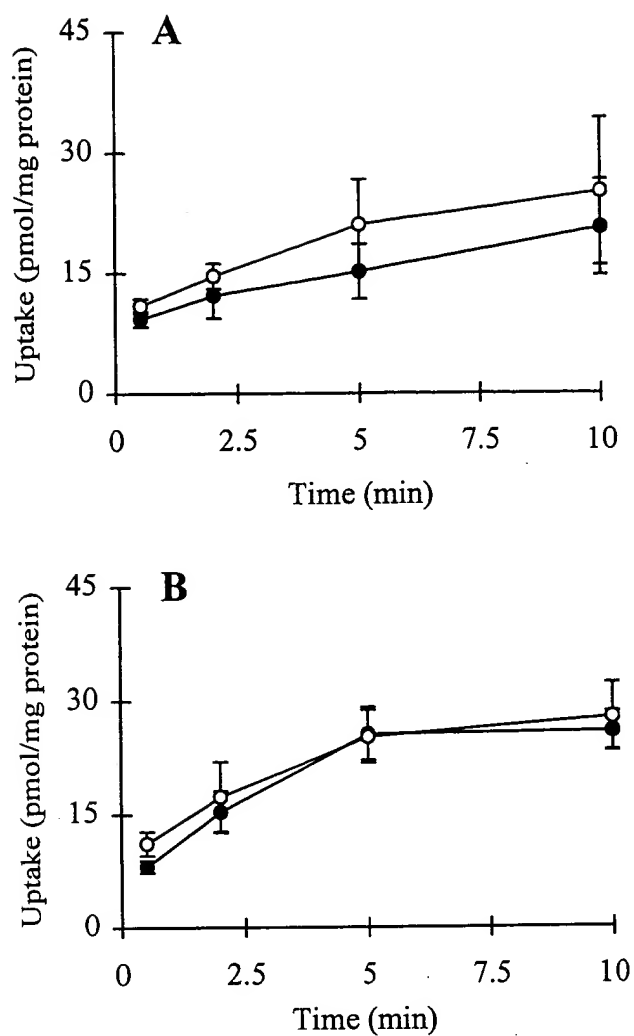


Figure 5-2. Cumulative uptake of $[^{14}\text{C}]$ salicylate (1 μM) in standard buffer (closed symbols) and Ca^{2+} -free buffer (open symbols) in hepatocyte monolayers cultured for 3 hr (A) and hepatocytes cultured in a sandwich configuration for 96 hr (B).

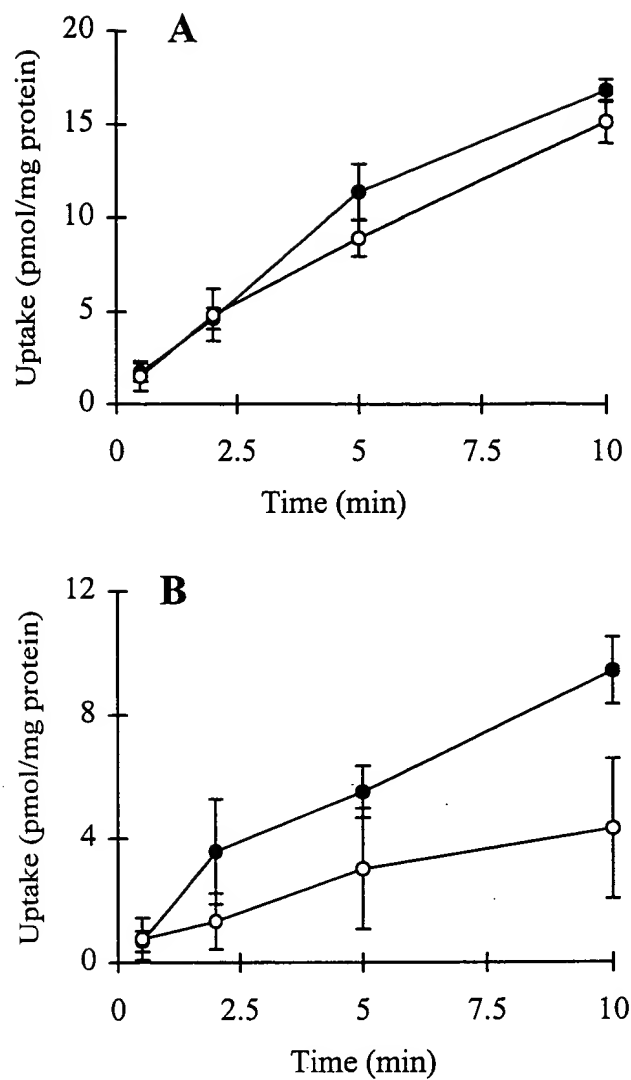


Figure 5-3. Cumulative uptake of $[^3\text{H}]$ methotrexate (1 μM) in standard buffer (closed symbols) and Ca^{2+} -free buffer (open symbols) in hepatocyte monolayers cultured for 3 hr (A) and hepatocytes cultured in a sandwich configuration for 96 hr (B).

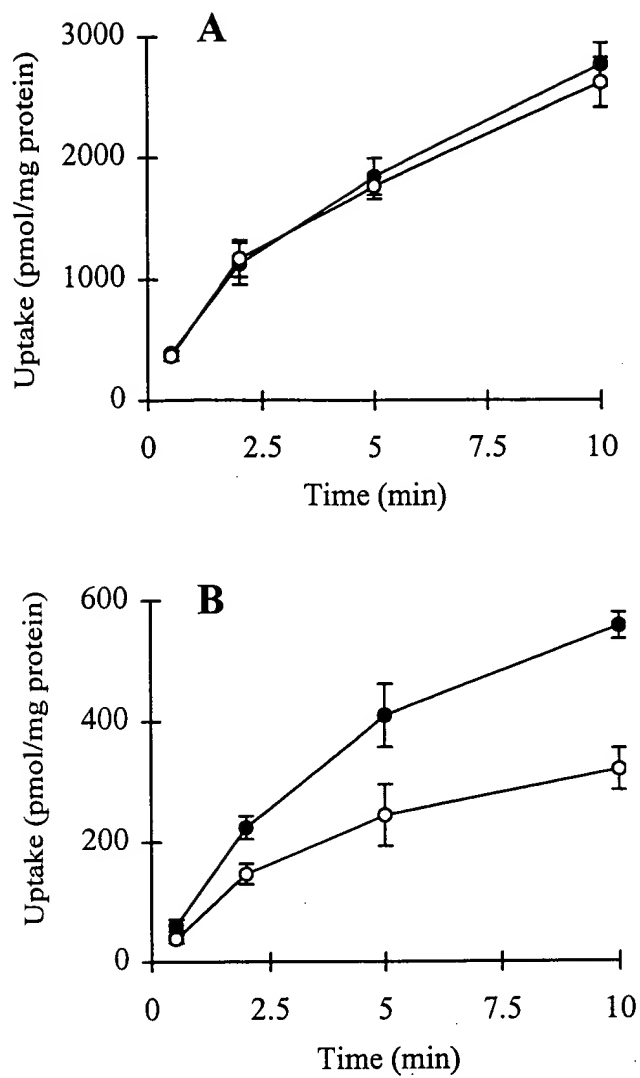


Figure 5-4. Cumulative uptake of $[^3\text{H}][\text{D-pen}^{2,5}]\text{enkephalin}$ (15 μM) in standard buffer (closed symbols) and Ca^{2+} -free buffer (open symbols) in hepatocyte monolayers cultured for 3 hr (A) and hepatocytes cultured in a sandwich configuration for 96 hr (B).

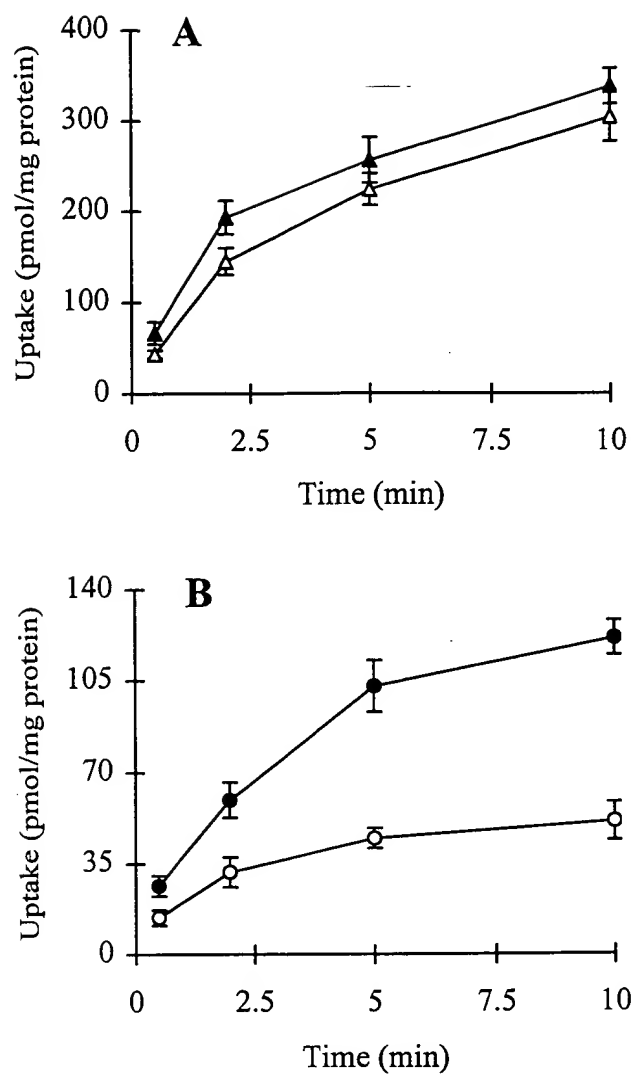


Figure 5-5. Cumulative uptake of $[^3\text{H}]$ taurocholate (1 μM) in standard buffer (closed symbols) and Ca^{2+} -free buffer (open symbols) in hepatocyte monolayers cultured for 3 hr (A) and hepatocytes cultured in a sandwich configuration for 96 hr (B).

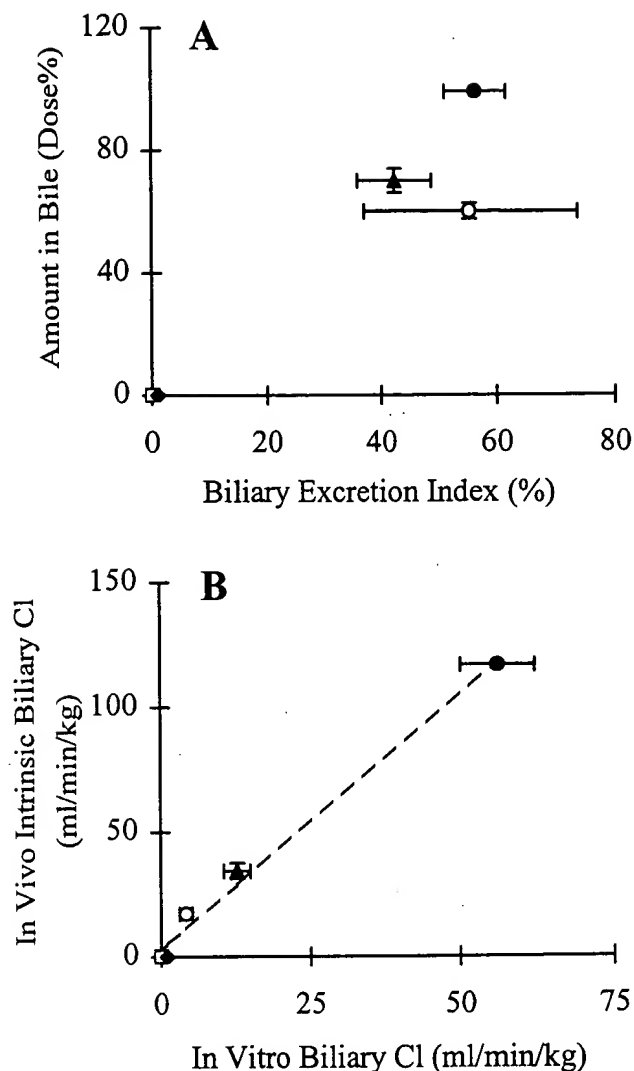


Figure 5-6. Relationship between the percentage of the dose excreted in rat bile *in vivo* and the Biliary Excretion Index (A), and *in vivo* intrinsic biliary clearance and *in vitro* biliary clearance (B), in 96-hr sandwich-cultured hepatocytes for the following model substrates: inulin (□), salicylate (◆), methotrexate (○), [D-pen^{2,5}]enkephalin (▲), and taurocholate (●). The Biliary Excretion Index was calculated from the 10-min cumulative uptake data (Figures 1-5) based on Equation 3. The *in vivo* intrinsic biliary clearance was calculated from Equation 2 based on *in vivo* biliary clearance values from the literature. The *in vitro* biliary clearance was calculated from Equation 4. The broken line is the fit of a linear regression equation to the data.

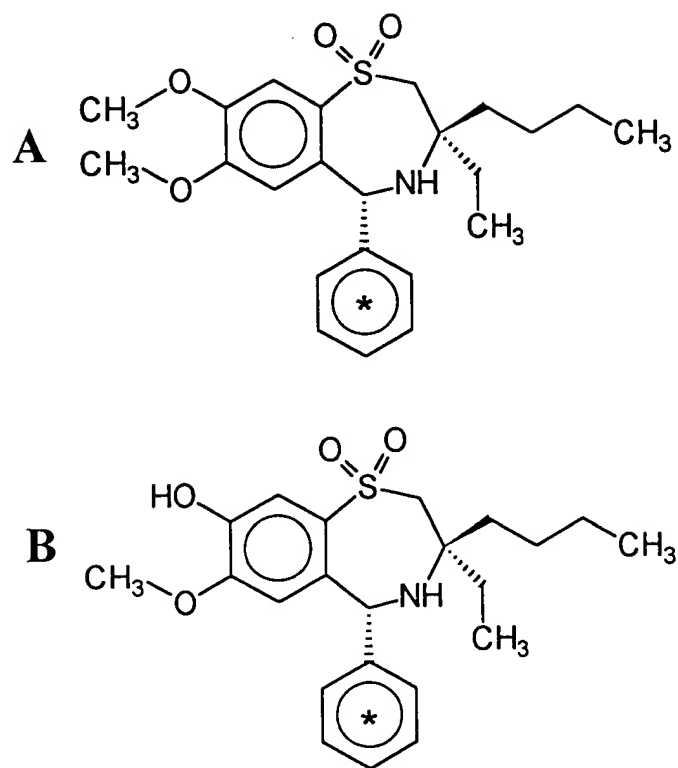


Figure 5-7. Chemical structure of 264W94 (A) and 2169W94 (B). A asterisk sign indicates position of ^{14}C incorporated uniformly.

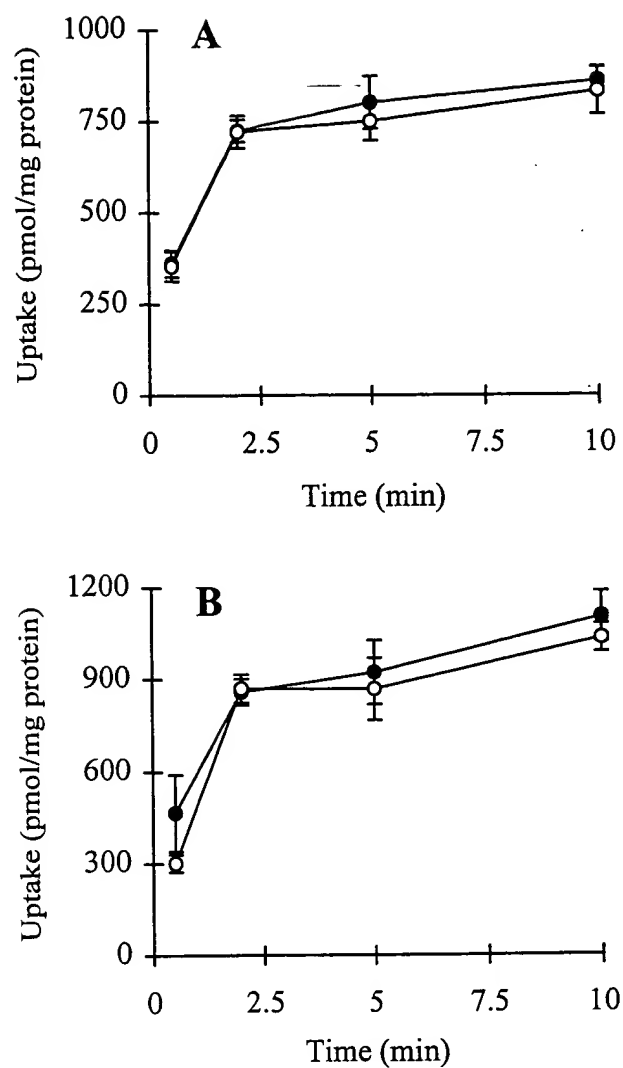


Figure 5-8. Cumulative uptake of $[^3\text{H}]264\text{W94}$ (3 μM) in standard buffer (closed symbols) and Ca^{2+} -free buffer (open symbols) in hepatocyte monolayers cultured for 3 hr (A) and hepatocytes cultured in a sandwich configuration for 96 hr (B).

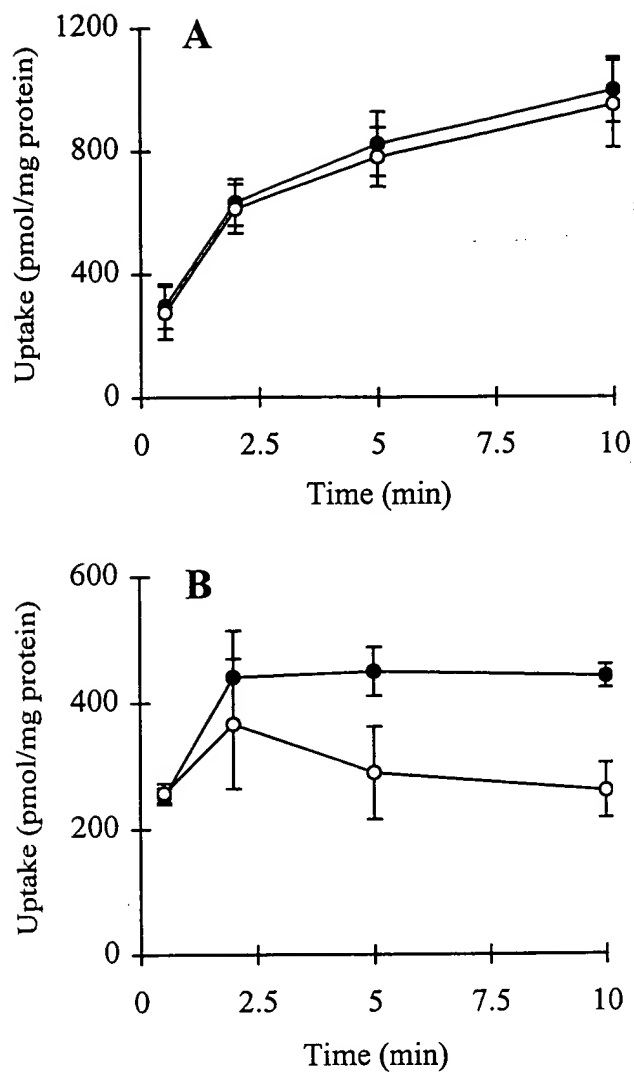


Figure 5-9. Cumulative uptake of $[^3\text{H}]2169\text{W94}$ (3 μM) in standard buffer (closed symbols) and Ca^{2+} -free buffer (open symbols) in hepatocyte monolayers cultured for 3 hr (A) and hepatocytes cultured in a sandwich configuration for 96 hr (B).

CHAPTER 6

CONCLUSIONS

The focus of this dissertation research was to establish a novel *in vitro* model to study *in vivo* biliary excretion. Evaluation of *in vivo* biliary excretion is important to predict the pharmacologic and toxicologic effects of drugs, variations in drug disposition in the presence of disease states, and drug-drug interactions. Characterization of biliary excretion also is critical in selection of drug candidates in discovery and development. Numerous *in vitro* preparations have been used to investigate biliary excretion processes (Oude Elferink *et al.*, 1995). However, existing methods may not always be applied to investigate human biliary excretion, and cannot be used to examine efficiently biliary excretion properties for a large number of drug candidates. Previous experiments demonstrated that primary rat hepatocytes cultured between two layers of gelled collagen maintain normal cell morphology, form extensive canalicular networks, and sustain liver-specific functions (Dunn *et al.*, 1991; LeCluyse *et al.*, 1994; 1996; Talamini *et al.*, 1997). Elaborate networks of bile canaliculi in sandwich-cultured hepatocyte monolayers are a structurally separate compartment that can accumulate secreted substrates. Therefore, the hepatocyte monolayers represent a potential *in vitro* model to study biliary excretion.

Substrates are excreted extensively into bile by active transport systems across the sinusoidal and/or canalicular membranes (Meijer *et al.*, 1983; Meijer, 1987; Oude Elferink *et al.* 1995). Thus, the application of sandwich-cultured hepatocytes as an *in vitro* model to study *in vivo* biliary excretion is possible only if the cultured hepatocytes are able to maintain the membrane transporters. Long-term primary rat hepatocytes cultured under conventional conditions cannot maintain membrane total transport activities (Liang *et al.*, 1993; Kukongviriyapan and Stacey, 1989). Hepatocytes cultured in a sandwich configuration maintain certain liver-specific functions such as albumin synthesis and some P450 induction

response. The maintenance of hepatobiliary transport activities in sandwich-cultured hepatocytes was investigated in this dissertation project.

In order to study biliary excretion in sandwich-cultured hepatocytes, quantitation of substrate excreted into the bile canaliculi in the monolayers was required. For fluorescent substrates, the amount of substrate in the canalicular networks in the monolayers can be quantitated by digital image analysis with fluorescence microscopy (Boyer and Soroka, 1995). However, most drugs are not fluorescent. In order to utilize the hepatocyte monolayers to study hepatobiliary transport processes, it was essential to develop a quantitative method that does not require fluorescent substrate. In this dissertation research, a quantitative method was proposed to assess the amount of substrate in the canalicular networks by determining the differential cumulative uptake of substrate in hepatocyte monolayers pre-incubated in standard buffer and Ca^{2+} -free buffer. A series of experiments was conducted to validate this proposed method. Furthermore, this method was used to examine quantitatively the re-establishment of functional polarity in sandwich-cultured hepatocytes, and to investigate the disposition of taurocholate in the long-term sandwich-cultured hepatocytes. Finally, the utility of this *in vitro* model to study *in vivo* biliary excretion was evaluated by examining the relationship between *in vitro* and *in vivo* biliary excretion of drugs and drug metabolites.

The hypothesis underlying this dissertation research was that primary rat hepatocytes cultured in a collagen-sandwich configuration can be utilized to study hepatobiliary disposition and biliary excretion. This hypothesis was tested by a series of experiments described below:

1. Examine the expression and the activity of Ntcp in cultured hepatocytes (4 hr and 96-120 hr) under conventional conditions or in a sandwich configuration. This study was designed to test the hypothesis that hepatocytes cultured in a sandwich configuration maintain the expression and activity of the hepatic uptake transport system.
2. Examine the expression and the activity of cMOAT in cultured hepatocytes (4 hr and 96-120 hr) in a sandwich configuration. This study was designed to test the hypothesis that hepatocytes cultured in a sandwich configuration maintain the expression and activity of biliary excretion transport systems.
3. Establish and validate a quantitative method to determine substrate in canalicular networks in sandwich-cultured hepatocytes. The substrate in the canalicular networks was determined by differential cumulative uptake in the monolayers pre-incubated in standard buffer and Ca^{2+} -free buffer. The cholephilic compound taurocholate, and the non-cholephilic compound salicylate, were utilized to validate this method. Three hypotheses involved in this quantitative method were tested.
 - (1) Ca^{2+} depletion disrupts the barrier function of tight junctions. This hypothesis was tested by the localization of ruthenium red staining in the monolayers incubated in standard buffer or Ca^{2+} -free buffer examined with electron microscopy.
 - (2) Substrate in the canalicular space and in the extracellular space diffuses rapidly via a paracellular pathway based on favorable concentration gradients during Ca^{2+} depletion. This hypothesis was tested by the localization of carboxydichlorofluorescein and rhodamine-dextran in the monolayers incubated

in standard buffer or Ca^{2+} -free buffer examined with confocal fluorescence microscopy.

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- (3) Ca^{2+} depletion does not change membrane transport properties. This hypothesis was tested by examining the cumulative uptake of taurocholate in freshly isolated hepatocytes, which lack intact canalicular networks, in standard buffer or Ca^{2+} -free buffer.
 4. Examine the re-establishment of functional polarity in sandwich-cultured hepatocytes. This study was designed to examine quantitatively the time-course of re-establishment of functional polarity in the cultured hepatocytes.
 5. Evaluate the disposition kinetics of substrate in long-term sandwich-cultured hepatocytes. This study was designed to test the hypothesis that long-term sandwich-cultured hepatocytes can be used to study hepatobiliary disposition.
 6. Assess the relationship between biliary excretion in long-term sandwich-cultured hepatocytes and *in vivo*. This study was designed to test the hypothesis that sandwich-cultured hepatocytes can be employed as an *in vitro* model system to study and predict biliary excretion *in vivo*.

Expression and Activity of Ntcp in Long-Term Sandwich-Cultured Rat Hepatocytes

Primary rat hepatocytes cultured between two layers of gelled collagen maintain normal morphology, form extensive canalicular networks, and sustain liver-specific functions (Dunn *et al.*, 1989; 1991; LeCluyse *et al.*, 1994). This study (Chapter 2) was undertaken to test the hypothesis that long-term (96-120 hr) cultured hepatocytes in a sandwich configuration maintain the expression and activity of hepatic uptake transport systems.

Several transport systems on the sinusoidal membrane for hepatic uptake of substrates have been characterized (Oude Elferink *et al.*, 1995). Among them, Ntcp is the best characterized system and represents typical liver-specific uptake. Thus, Ntcp was chosen as the model transporter for examination in this study. Hepatocytes isolated from male Wistar rats (230-280 g) were cultured on a simple collagen film (conventional condition), on a substratum of gelled collagen, or between two layers of gelled collagen. Hepatocyte morphology was examined in these cultures at 4 and 120 hr. The expression and activity of Ntcp was determined by immunoblot analysis and taurocholate uptake properties, respectively. At 120 hr, monolayer integrity had deteriorated in simple collagen cultures. In contrast, cell morphology was preserved and extensive bile canalicular networks were formed in hepatocytes maintained in a sandwich configuration. Immunoblot analysis indicated that at 120 hr, Ntcp (~51 kDa; Stieger *et al.*, 1994) expression in hepatocytes cultured in a sandwich configuration could be detected, but Ntcp expression in hepatocytes cultured on a simple collagen film could not be detected. At 120 hr, [^3H]taurocholate accumulation at 5 min in hepatocytes cultured on a simple collagen film, on a substratum of gelled collagen, and in a sandwich configuration was ~13%, 20% and 35% of 4-hr levels, respectively, and occurred predominately by a Na^+ -dependent mechanism. The initial taurocholate uptake rate versus concentration (1-200 μM) profile was best described by a combined Michaelis-Menten and first-order function. In all cases, values for the estimated apparent Michaelis-Menten constant (K_m) were comparable for 4- and 120- hr hepatocytes (32-41 μM). In contrast, values for the maximal uptake capacity (V_{max}) of hepatocytes cultured on a simple collagen film, on gelled collagen and in a sandwich configuration were ~5, 6 and 14% of the values at 4 hr, respectively; values for the first-order rate constant were 5-, 3- and 2-fold

lower, respectively. Together, these data demonstrated that long-term hepatocytes cultured in a collagen-sandwich configuration reestablish normal hepatocyte morphology and retain bile acid uptake activity. These results also suggested that taurocholate may be retained in the canalicular networks because cumulative uptake (35%) was greater than predicted based on V_{\max} values (14%).

Expression and Activity of cMOAT in Long-Term Sandwich-Cultured Rat Hepatocytes

Based on the results of studies discussed in Chapter 2, hepatocytes cultured in a sandwich configuration maintain hepatic uptake transport activities. A subsequent experiment (Chapter 3) was performed to test the hypothesis that long-term sandwich-cultured hepatocytes maintain the expression and activity of biliary excretion transport systems. At least four ATP-dependent primary active transport systems exist on the canalicular membrane (Oude Elferink *et al.*, 1995). Among these transport systems, cMOAT is a well-characterized and important transporter for biliary excretion of organic anions (Paulusma *et al.*, 1996). Thus, it was chosen as the model transporter for evaluation of biliary excretion properties in sandwich-cultured hepatocytes. Hepatocytes isolated from male Wistar rats (230-280 g) were cultured on a substratum of gelled collagen or between two layers of gelled collagen. The expression and activity of the canalicular transport protein, cMOAT, were examined in these cultures at 4 and 120 hr by immunoblot analysis and the localization of carboxydichlorofluorescein, respectively. Localization of carboxydichlorofluorescein, a cMOAT substrate (Kitamura *et al.*, 1990), was examined with fluorescence microscopy. Immunoblot analysis demonstrated that a significant amount of cMOAT (~190 kDa) was maintained in hepatocytes cultured in a sandwich configuration for 120 hr compared to

hepatocytes cultured for 4 hr. A slight increase (10-15 kDa) in the molecular weight of cMOAT was observed in the 120-hr sandwich-cultured hepatocytes, presumably due to the alteration of posttranslational processing of this protein (Trauner *et al.*, 1997). Carboxydichlorofluorescein was excreted into and concentrated in the bile canalicular lumen of 120-hr sandwich-cultured hepatocytes, resulting in formation of fluorescent networks. These data indicated that hepatocytes cultured in a collagen sandwich configuration for 120 hr retain significant cMOAT expression and functional activity. Together, these results demonstrated that long-term sandwich-cultured hepatocytes maintain the transport activity necessary for biliary excretion of some substrates.

Establishment of a Quantitative Method for Determination of Excreted Substrate in Canalicular Networks of Sandwich-Cultured Rat Hepatocytes

Long-term sandwich-cultured hepatocytes develop extensive bile canalicular networks and maintain hepatic uptake and biliary excretion activities (Chapter 2 and 3). These hepatocyte monolayers represent a potential *in vitro* model to study hepatobiliary transport. However, quantitation of non-fluorescent substrate in the canalicular networks (*i.e.*, biliary excretion in the hepatocyte monolayers) has not been reported. The objective of the present investigation was to establish a quantitative method to assess biliary excretion in the sandwich-cultured hepatocytes. Hepatocytes isolated from male Wistar rats (230-280 g) were cultured on a substratum of gelled collagen or between two layers of gelled collagen. The proposed quantitative method was based on 3 assumptions or hypotheses, which were tested individually.

Hypothesis I: Tight junctions in sandwich-cultured hepatocytes are the diffusional barrier between the canalicular lumen and the extracellular space; Ca^{2+} depletion rapidly disrupts the barrier function of the tight junctions. This hypothesis was tested by the localization of ruthenium red staining in the hepatocyte monolayers incubated in standard buffer or Ca^{2+} -free buffer examined with electron microscopy (Chapter 4). In standard buffer, ruthenium red staining was visible on the basolateral membrane but not on the canalicular membrane. In contrast, in Ca^{2+} -free buffer, ruthenium red staining was visible on the basolateral and canalicular membranes. This electron microscopy study demonstrated that tight junctions are the diffusional barrier between the canalicular lumen and the extracellular space; Ca^{2+} depletion increased the permeability of the tight junctions.

Hypothesis II: Ca^{2+} depletion disrupts the integrity of canalicular networks in sandwich-cultured hepatocytes and enables substrate to translocate rapidly between the canalicular and extracellular spaces. This hypothesis was tested by the localization of carboxydichlorofluorescein (canalicular space marker) and rhodamine-dextran (MW 10 kDa, extracellular space marker) in sandwich-cultured hepatocytes that were incubated in standard buffer or Ca^{2+} -free buffer. The fluorescence was examined with confocal fluorescence microscopy (Chapter 3). In standard buffer, carboxydichlorofluorescein was concentrated in the canalicular spaces; rhodamine-dextran was localized in the incubation medium and could not penetrate into the canalicular spaces. In contrast, after the monolayers were incubated in Ca^{2+} -free buffer for 2-5 min, fluorescence of carboxydichlorofluorescein was not visible; rhodamine-dextran penetrated into the canalicular spaces. These results demonstrated that after incubation of sandwich-cultured hepatocytes in Ca^{2+} -free buffer for 2-5 min, the

integrity of the canalicular lumen was disrupted, and substrate diffused readily between the canalicular lumen and the extracellular space, based on favorable concentration gradients.

Hypothesis III: Ca^{2+} depletion does not change membrane transport properties. This hypothesis was tested by measuring the cumulative uptake of taurocholate in suspensions of freshly isolated hepatocytes in standard buffer or Ca^{2+} -free buffer (Chapter 4). Freshly isolated hepatocytes were chosen as the logical model system for this investigation because these hepatocytes maintain membrane transport properties but lack intact canalicular networks and functional tight junctions (Talamini *et al.*, 1997). The initial uptake rate and cumulative uptake of [^3H]taurocholate in freshly isolated hepatocytes was not statistically different in standard buffer and Ca^{2+} -free buffer, demonstrating that taurocholate transport was not significantly affected by depletion of extracellular Ca^{2+} .

Based upon these 3 assumptions, two quantitative methods were proposed. The rationale of these two methods was that sandwich-cultured hepatocyte monolayers are composed of two separate compartments in standard buffer: cytosol and canalicular lumen. After Ca^{2+} depletion, the permeability of the tight junctions increases and substrate can diffuse readily between the canalicular lumen and the extracellular space (paracellular pathway). In essence, the two-compartment model becomes a one-compartment model in which only the cytosolic compartment exists in the hepatocyte monolayers incubated in Ca^{2+} -free buffer. When hepatocyte monolayers are incubated in standard buffer with a cholephilic substrate, substrate in the medium is taken up by hepatocytes and excreted into the bile canalicular networks. In standard buffer, the excreted substrate remains in the canalicular compartment; in Ca^{2+} -free buffer, the substrate in the canalicular compartment diffuses back into the incubation medium. Thus, in standard buffer, the cumulative uptake of a substrate in

the long-term sandwich-cultured hepatocytes represents the amount of substrate in the cytosolic and canalicular compartments; in Ca^{2+} -free buffer, the cumulative substrate uptake represents the amount of substrate in the cytosolic compartment. Because the transport activity is independent of extracellular Ca^{2+} concentrations, the amount of substrate in the cytosolic compartment is the same in standard buffer and Ca^{2+} -free buffer. Therefore, substrate in the canalicular compartment in the monolayers can be estimated by the difference in cumulative substrate uptake in the presence (standard buffer) and absence (Ca^{2+} -free buffer) of extracellular Ca^{2+} .

Based on similar principles, another approach to determine substrate in the canalicular spaces is to measure the differential efflux of substrate in the presence and absence of extracellular Ca^{2+} . When hepatocyte monolayers are preincubated in standard buffer with a cholephilic substrate, substrate in the medium is taken up by hepatocytes and excreted into the bile canalicular networks. After preloading the hepatocytes, the incubation medium containing substrate is removed and fresh medium that does not contain substrate is added. If the fresh medium does not contain extracellular Ca^{2+} , tight junction permeability increases enabling substrate in the canaliculi to diffuse rapidly into the medium. In contrast, if the fresh medium contains extracellular Ca^{2+} , substrate in the canaliculi will be retained there or may slowly diffuse into the medium. Thus, the appearance of substrate in the medium (efflux) in Ca^{2+} -free buffer will be greater than in standard buffer; this difference in efflux represents the amount of substrate in the canalicular networks.

It has been demonstrated that a fluorescent bile acid, fluorescein-taurocholate, was excreted into and concentrated in the canalicular networks in 120-hr sandwich-cultured hepatocytes (Chapter 3), indicating that the canalicular bile acid transporter was functional.

In the present studies, [^3H]taurocholate was used as a model substrate to validate the proposed methods to quantitate substrate in the canalicular networks in sandwich-cultured hepatocytes (Chapter 3). Salicylate was employed as a negative control. Salicylate undergoes hepatic metabolism and both unchanged and metabolized salicylate are extensively excreted in urine (Nelson *et al.*, 1966; Laznicek and Laznickova, 1994); data indicate that salicylate can penetrate the plasma membrane, but neither its parent form nor metabolites is excreted in bile. Biliary excretion in the monolayers was examined with cumulative uptake and efflux studies. As expected, the 10-min cumulative uptake of [^3H]taurocholate in 120-hr sandwich-cultured hepatocytes was significantly higher in standard buffer than in Ca^{2+} -free buffer. As a control study, the 10-min cumulative uptake of [^3H]taurocholate in 4-hr cultured hepatocytes which do not maintain intact bile canaliculi was only slightly higher in standard buffer than in Ca^{2+} -free buffer. After [^3H]taurocholate was pre-loaded in 120-hr sandwich-cultured hepatocytes, taurocholate efflux was greater in Ca^{2+} -free buffer than in standard buffer. In short-term cultured hepatocytes, no difference in taurocholate efflux was observed, suggesting that no significant biliary excretion occurred, or that the excreted taurocholate could not be retained in the canalicular lumen due to leakage. Salicylate did not show any difference in cumulative uptake or efflux in standard buffer and Ca^{2+} -free buffer. These studies demonstrated that the differential cumulative uptake or efflux of substrate in standard buffer and Ca^{2+} -free buffer can be utilized to determine the amount of substrate in the canalicular networks in sandwich-cultured hepatocytes.

One disadvantage of the efflux method is that differential efflux may underestimate the actual amount of substrate in the canalicular networks because, in the absence of extracellular Ca^{2+} , substrate that has diffused out of the canalicular spaces into the medium is

available for reuptake by hepatocytes. However, in cumulative uptake studies, substrate concentrations in the medium remain relatively constant and uptake should be the same in the presence and absence of extracellular Ca^{2+} . Measurement of cumulative substrate uptake in standard buffer and Ca^{2+} -free buffer is a more accurate method than the efflux method to quantitate the amount of substrate in the canalicular networks. Thus, the cumulative uptake method was utilized in subsequent studies.

Although Ca^{2+} depletion did not interfere with transport properties of the model substrate taurocholate, this does not rule out the possibility that Ca^{2+} depletion may interfere with the transport activities of other substrates (Petzinger and Frimmer, 1988). Therefore, in this dissertation research, after incubation of the monolayers in standard buffer or in Ca^{2+} -free buffer for 10 min, all cumulative uptake studies were conducted in standard buffer to prevent potential interfering effects of Ca^{2+} depletion on substrate transport in the hepatocyte monolayers. This approach was based on the hypothesis that the functional integrity of tight junctions, which had been disrupted after incubation of the monolayers in Ca^{2+} -free buffer, could not be reestablished during the short duration of transport studies. In order to test this hypothesis, canalicular morphology and cumulative taurocholate uptake were examined at designated times during incubation in standard buffer after monolayers had been incubated for 10 min in Ca^{2+} -free buffer (Chapter 4). The smaller canalicular diameter due to Ca^{2+} depletion did not appear to change noticeably during exposure of the monolayers to standard buffer for 10 min. The 10-min cumulative [^3H]taurocholate uptake remained significantly lower than control values (monolayers that were not incubated in Ca^{2+} -free buffer), even after incubation of the monolayers in standard buffer for 60 min.

Re-establishment of Functional Polarity in Cultured Rat Hepatocytes

Freshly isolated hepatocytes lose cell polarity and do not maintain intact bile canalicular networks (Graf and Boyer, 1990). Previous studies suggested that hepatocytes cultured in a sandwich configuration for 120 hr re-establish functional polarity (Chapter 2). However, the optimal time after hepatocytes have been plated for utilization of sandwich-cultured hepatocyte monolayers for biliary excretion studies had not been determined. In order to address this question, the time-course of re-establishment of functional polarity in the hepatocyte monolayers was examined (Chapter 3). Hepatocytes isolated from male Wistar rats (230-280 g) were cultured on a substratum of gelled collagen or between two layers of gelled collagen. Taurocholate was employed as a model substrate in this study. The Biliary Excretion Index, defined as the percentage of retained substrate in the canalicular networks, was utilized to evaluate quantitatively the functional polarity. The Biliary Excretion Index of taurocholate increased from ~8% in hepatocytes cultured for 4 hr to ~60% in sandwich-cultured hepatocytes at 72 hr. Thereafter, the Biliary Excretion Index of taurocholate appeared to reach a plateau. These results suggested that hepatocytes cultured in a sandwich configuration re-establish functional polarity within 72 hr in culture, and can be used to study biliary excretion. In this dissertation project, biliary excretion studies were conducted in hepatocytes that had been cultured in a sandwich configuration for 96-120 hr.

Hepatobiliary Disposition of Taurocholate in Sandwich-Cultured Rat Hepatocytes

Previous work has indicated that biliary excretion of a substrate in sandwich-cultured hepatocytes can be quantitated by measuring differential cumulative uptake of substrate in

the presence and absence of extracellular Ca^{2+} (Chapter 3). The objective of the present study was to analyze taurocholate disposition in the hepatocyte monolayers utilizing a kinetic modeling approach (Chapter 4). Sandwich-cultured rat hepatocytes were prepared by maintaining hepatocytes isolated from Wistar rats (250-280g) on Petri dishes pre-coated with collagen gel for 24 hr before overlaying with collagen gel solution. Cumulative uptake of [^3H]taurocholate (1-100 μM) was quantitated in 96-hr sandwich-cultured hepatocytes pre-incubated in standard buffer or Ca^{2+} -free buffer. Seventeen different kinetic models incorporating 1 or 2 compartments with first-order and/or Michaelis-Menten uptake and elimination processes were examined to select the model that best described the cumulative uptake of taurocholate. The differential equations derived for each model were solved simultaneously with nonlinear least-squares regression to describe the cumulative taurocholate uptake in the presence and absence of extracellular Ca^{2+} . The model that best described the cumulative taurocholate uptake in sandwich-cultured hepatocyte monolayers consisted of cytosolic and bile compartments in standard buffer, but only a cytosolic compartment in Ca^{2+} -free buffer. Michaelis-Menten processes best described taurocholate uptake ($K_m = 28.0 \pm 3.6 \mu\text{M}$, $V_{\max} = 1.19 \pm 0.07 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$) and biliary excretion ($K_m = 1.03 \pm 0.35 \text{ nmol} \cdot \text{mg protein}^{-1}$, $V_{\max} = 1.19 \pm 0.07 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$) processes. The model structure was consistent with the results obtained from other studies utilizing electron microscopy and confocal fluorescence microscopy techniques in this dissertation project. The estimated kinetic parameters are comparable with values reported in the literature (Boyer and Meier, 1990). These results demonstrated that sandwich-cultured hepatocytes represent a novel *in vitro* model to study hepatobiliary disposition.

Relationship Between Biliary Excretion in Sandwich-Cultured Rat Hepatocytes and *In Vivo* in Rats

Previous studies indicated that biliary excretion is maintained in sandwich-cultured hepatocytes and can be quantitated by the differential cumulative uptake of substrate in monolayers pre-incubated in standard buffer and Ca^{2+} -free buffer (Chapter 3). In order to determine whether the biliary excretion in sandwich-cultured hepatocytes can be used to predict *in vivo* biliary excretion, the present study was designed to investigate the relationship between biliary excretion in long-term sandwich-cultured rat hepatocytes and *in vivo* in rats (Chapter 5). Sandwich-cultured hepatocytes were prepared by maintaining hepatocytes isolated from Wistar rats (250-280g) on Petri dishes pre-coated with collagen gel for 24 hr before overlaying with collagen gel solution. Biliary excretion of five model substrates (inulin, salicylate, methotrexate, [D-Pen^{2,5}]enkephalin, and taurocholate) in 96-hr cultured hepatocytes was determined by differential cumulative uptake of substrate in the monolayers pre-incubated in standard buffer and Ca^{2+} -free buffer. The relationship between the percentage of the dose excreted in bile *in vivo* in rats and the Biliary Excretion Index, as well as the relationship between *in vivo* intrinsic biliary clearance and *in vitro* biliary clearance, were examined. The percentage of the dose excreted in bile in 1 hr in rats for inulin, salicylate, methotrexate, [D-Pen^{2,5}]enkephalin, and taurocholate was ~0% (Eriksson *et al.*, 1975), ~0% (Nelson *et al.*, 1966; Laznicek and Laznickova, 1994), 50-60% (Bremnes *et al.*, 1989; Masuda *et al.*, 1997), ~70% (Chen and Pollack, 1997), and ~100% (Inoue *et al.*, 1985), respectively. The Biliary Excretion Index of inulin, salicylate, methotrexate, [D-Pen^{2,5}]enkephalin, and taurocholate was ~0%, ~0%, $55.4 \pm 18.3\%$, $42.4 \pm 6.5\%$, and $56.4 \pm$

5.2%, respectively. In general, the Biliary Excretion Index was consistent with the percentage of the dose excreted in bile from *in vivo* experiments. However, the Biliary Excretion Index could not differentiate between compounds that were “highly” excreted in bile (*e.g.*, methotrexate, [D-Pen^{2,5}]enkephalin) and compounds that undergo “extensive” biliary excretion (*e.g.*, taurocholate). The *in vivo* intrinsic biliary clearance of inulin, salicylate, methotrexate, [D-Pen^{2,5}]enkephalin, and taurocholate was 0.035 (Eriksson *et al.*, 1975), ~0 (Nelson *et al.*, 1966; Laznicek and Laznickova, 1994), 12.1 (Masuda *et al.*, 1997), 18.5 (Chen and Pollack, 1997), and 29.8 ml/min/kg of body weight (Inoue *et al.*, 1985), respectively. The *in vitro* biliary clearance of inulin, salicylate, methotrexate, [D-Pen^{2,5}]enkephalin, and taurocholate, calculated as the ratio of the amount excreted into the bile canalicular networks and the area under the incubation medium concentration-time profile, was ~0, ~0, 4.5 ± 1.1 , 12.6 ± 2.2 , and 56.2 ± 6.0 ml/min/kg, respectively. The *in vitro* biliary clearance of these 5 model substrates correlated well with their *in vivo* intrinsic biliary clearance ($R^2 = 0.9865$). Calculation of the *in vivo* intrinsic and *in vitro* biliary clearance enabled differentiation between compounds that are highly and extensively excreted in bile. This study suggested that long-term sandwich-cultured hepatocytes represent a useful *in vitro* model to predict and study *in vivo* biliary excretion.

A secondary objective of this specific aim was to assess the utility of this *in vitro* model to study *in vivo* biliary excretion of drug metabolites. 264W94 and its O-demethylated metabolite 2169W94 were chosen as model substrates. The biliary excretion of these 2 compounds in rats and in 96-hr sandwich-cultured rat hepatocytes was examined. The model compound 264W94 was not excreted in bile, either *in vivo* or *in vitro*. However, the glucuronide conjugate of 2169W94, was excreted into bile *in vitro* when 2169W94, but not

264W94, was incubated with the monolayers; 2169W94 glucuronide undergoes extensive biliary excretion after administration of 2169W94 *in vivo*. Biliary excretion in long-term sandwich-cultured rat hepatocytes correlates with *in vivo* biliary excretion. The study of biliary excretion of metabolites in the monolayers requires consideration of the status of metabolic activities.

Future Directions

This dissertation research represents the first attempt to study hepatobiliary transport processes in long-term sandwich-cultured primary rat hepatocytes. Although this *in vitro* model is intriguing and promising in studying the hepatobiliary disposition and biliary excretion of substrates, more research is required in order to realize the full potential, and to reveal the capabilities and limitations, of this *in vitro* model system. Based on the results of this dissertation research, future studies with this *in vitro* model should focus on further examination of the membrane transport systems, optimization of the culture conditions to maintain membrane transport activities, assessment of *in vitro* and *in vivo* correlation of biliary excretion for a variety of substrates, and application of this *in vitro* technique to study human biliary excretion.

Numerous transport systems have been characterized on the sinusoidal and canalicular membrane. In this dissertation research, hepatic uptake and biliary excretion activity were demonstrated with Ntcp and cMOAT, respectively. In order to characterize the membrane transport properties of the hepatocyte monolayers, the expression and activity of other transport systems such as oatp 1 (Jacquemin *et al.* 1991; 1994; Buscher *et al.*, 1987) and oct 1 (Grundemann *et al.*, 1994) on the sinusoidal membrane, and P-glycoprotein

(Spoelstra *et al.*, 1994) and Sister of P-glycoprotein (Childs *et al.*, 1996; Muller *et al.*, 1996) on the canalicular membrane should be examined. In addition, whether maintenance of the transport systems is due to sustained transcription or translation of the transport proteins in the sandwich-cultured hepatocytes should be investigated.

The culture conditions should be systematically evaluated to define the optimal conditions for hepatobiliary transport studies. The culture conditions used in this dissertation research were optimized based on hepatocyte and canalicular morphology. However, under the current culture conditions, taurocholate uptake activity in 120-hr sandwich-cultured hepatocytes deteriorated considerably compared to 4-hr cultured hepatocytes, although Ntcp could be detected in 120-hr sandwich-cultured hepatocytes. Therefore, future studies should examine the effects of medium composition and extracellular matrix on the expression and activity of each known membrane transport protein. As the metabolic activities, particularly the phase I metabolic activities, deteriorate in long-term sandwich-cultured hepatocytes, culture conditions will need to be optimized to maintain both hepatic transport and phase I and phase II enzyme activities in order to study the biliary excretion of metabolites. In addition, current methods to quantitate biliary excretion need to be optimized. In the present study, the biliary excretion in sandwich-cultured hepatocytes was measured by pre-incubation of the monolayers in standard buffer or Ca^{2+} -free buffer for 10 min prior to initiation of cumulative substrate uptake in standard buffer. Results of the present studies indicate that the permeability of tight junctions increased within 5 min after Ca^{2+} removal (Chapter 4). Future studies should examine whether shorter pre-incubation times in Ca^{2+} -free buffer can be utilized to minimize the potential effects of Ca^{2+} depletion on cell function.

In the present study, biliary excretion in sandwich-cultured hepatocytes expressed as apparent biliary clearance appeared to correlate to *in vivo* intrinsic biliary clearance. Future studies should examine a larger number of substrates possessing a variety of structural characteristics to assess the correlation between *in vitro* and *in vivo* biliary excretion, and the relationship between chemical structure and biliary excretion. In addition, experimental approaches should be developed to adapt this *in vitro* model to high throughput drug screening. A possible approach is first to identify a series of markers for hepatic uptake and biliary excretion transport systems and then to determine the inhibitory effect of drug candidates on the hepatic transport of these markers. Information regarding alterations in biliary excretion of the markers may indicate whether drug candidates are substrates for specific transporters, or if they can otherwise modulate the biliary excretion of compounds.

Finally, the utility of sandwich-cultured primary human hepatocytes to predict and study human biliary excretion should be examined. Recently, Kono *et al.* (1997) reported that sandwich-cultured human hepatocytes formed elaborate bile canalicular networks. Whether hepatobiliary transport systems are maintained in the human sandwich-cultured hepatocyte monolayers, and whether biliary excretion in sandwich-cultured human hepatocytes correlates with *in vivo* biliary excretion in humans, remains to be elucidated. In addition, it would be of interest to investigate species differences in biliary excretion, and the effects of disease states such as cirrhosis on the biliary excretion of endogenous compounds and xenobiotics, as well as the underlying mechanisms of biliary excretion with this *in vitro* model.

In summary, this dissertation project represents a significant advance in the development of cultured hepatocytes as an *in vitro* model system to study biliary excretion.

Results have demonstrated that (i) primary rat hepatocytes cultured in a collagen-sandwich configuration for 120 hr maintain functional hepatic membrane transport systems, including hepatic uptake and biliary excretion activities; (ii) Ca^{2+} depletion increases the permeability of tight junctions but does not alter hepatic membrane transport properties; (iii) substrate in the canalicular networks in sandwich-cultured hepatocytes can be quantitated by differential cumulative uptake in the monolayers pre-incubated in standard buffer and Ca^{2+} -free buffer; and (iv) sandwich-cultured hepatocytes can be utilized to study hepatobiliary disposition and to predict *in vivo* biliary excretion.

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APPENDIX

DATA SUMMARY

Chapter 2

Percent of Radioisotope Remaining After Each Wash Step (Figure 2-1, n=3)

Wash Number	Sandwiched Cultures		Cell-Free Double-Layer Gel	
	Mean	SD	Mean	SD
0	100.00	0.00	100.00	0.00
1	31.24	2.59	4.33	1.48
2	27.69	2.15	0.93	0.09
3	26.53	2.05	0.31	0.18
4	25.85	1.91	0.22	0.09
5	25.35	1.85	0.15	0.10
6	25.01	1.83	0.13	0.06
7	24.66	1.76	0.08	0.06
8	24.36	1.67	0.08	0.02
9	24.03	1.61		
10	1.79	0.09		
11	0.68	0.04		
12	0.44	0.03		
13	0.32	0.02		
14	0.25	0.02		
15	0.21	0.02		
16	0.17	0.02		

Chapter 2

Temperature- and Sodium-Dependence of Taurocholate ($1 \mu\text{M}$) Uptake (pmol/mg protein) in Cultured Rat Hepatocytes (Figure 2-3, n=6-12)

A: Day 0, Gelled Substratum B: Day 0, Rigid Substratum

Time (min)	HBSS, 37°C		HBSS, 37°C	
	Mean	SD	Mean	SD
0.5	95.1	17.6	77.8	9.6
1	133.4	13.6	112.9	3.1
2	216.4	31.8	177.3	10.5
5	297.8	32.9	249.7	10.5

Time (min)	Na ⁺ -Free HBSS, 37°C		Na ⁺ -Free HBSS, 37°C	
	Mean	SD	Mean	SD
0.5	28.2	11.2	18.9	3.1
1	36.4	9.4	20.4	1.1
2	50.5	12.3	37.3	4.8
5	78.1	18.2	57.3	2.3

Time (min)	HBSS, 4°C		HBSS, 4°C	
	Mean	SD	Mean	SD
0.5	7.5	5.1	2.5	0.5
1	5.8	3.4	4.3	2.7
2	10.1	6.5	6.7	4.1
5	18.2	5.1	14.5	4.1

C: Day 5, Gelled SubstratumD: Day 5, Rigid Substratum

Time (min)	HBSS, 37°C	
	Mean	SD
0.5	13.7	4.4
1	22.3	5.8
2	39.4	11.9
5	60.4	17.5

	HBSS, 37°C	
	Mean	SD
0.5	8.5	1.9
1	16.1	1.3
2	25.8	2.1
5	40.4	8.3

Time (min)	Na ⁺ -Free HBSS, 37°C	
	Mean	SD
0.5	2.2	0.8
1	3.1	0.6
2	3.9	1.0
5	6.2	1.3

	Na ⁺ -Free HBSS, 37°C	
	Mean	SD
0.5	1.9	0.4
1	1.9	0.2
2	3.2	0.7
5	5.1	0.4

Time (min)	HBSS, 4°C	
	Mean	SD
0.5	1.4	0.4
1	1.1	0.3
2	1.4	0.4
5	3.4	0.4

	HBSS, 4°C	
	Mean	SD
0.5	0.7	0.2
1	0.9	0.2
2	1.3	0.4
5	2.3	0.3

E: Day 5, Sandwich Configuration

HBSS, 37°C

Time (min)	Mean	SD
0.5	20.4	5.7
1	31.4	12.0
2	62.1	9.4
5	103.7	10.2

Na⁺-Free HBSS, 37°C

Time (min)	Mean	SD
0.5		
1	2.8	0.4
2	3.3	0.6
5	6.0	0.5
	6.7	1.6

HBSS, 4°C

Time (min)	Mean	SD
0.5	0.7	0.4
1	1.1	0.4
2	2.4	0.9
5	3.6	0.3

Chapter 2

Linear Range of Taurocholate Initial Uptake (pmol/mg protein, Figure 2-4)

<u>Time (min)</u>	<u>1 μM</u>	<u>10 μM</u>	<u>50 μM</u>	<u>100 μM</u>	<u>200 μM</u>
0.25	13.7	128.6	307.5	431.4	428.5
0.5	30.0	223.3	566.7	781.3	1105.7
1	43.3	360.6	760.0	1142.6	1779.5
2	68.0	455.5	1398.5	1779.9	2063.2

Chapter 2

Initial Uptake Rate of Taurocholate (nmol/min/mg protein, Figure 2-5)

Day 0, Gelled substratum				Day 5, Gelled Substratum			
Conc.	Mean	SD	Simulated	Conc.	Mean	SD	Simulated
1	0.07	0.03	0.15	1	0.01	0.01	0.01
5	0.48	0.07	0.68	5	0.05	0.01	0.06
10	0.94	0.26	1.22	10	0.09	0.04	0.11
20	1.93	0.41	2.06	20	0.20	0.05	0.18
30	2.88	0.66	2.68	30	0.26	0.08	0.25
50	3.73	0.70	3.57	50	0.32	0.12	0.36
100	5.32	0.73	4.95	100	0.65	0.27	0.58
200	6.48	0.90	6.70	200	0.94	0.19	0.97

Day 5, Sandwich Configuration				Conc.	Day 5, Rigid Substratum		
Conc.	Mean	SD	Simulated	Conc.	Mean	SD	Simulated
1	0.03	0.02	0.02	1	0.02	0.00	0.01
5	0.11	0.03	0.11	5	0.04	0.00	0.04
10	0.20	0.09	0.21	10	0.06	0.05	0.07
20	0.35	0.14	0.36	20	0.12	0.02	0.13
30	0.52	0.07	0.48	30	0.10	0.02	0.18
50	0.61	0.24	0.67	50	0.24	0.17	0.25
100	1.09	0.26	1.03	100	0.48	0.13	0.41
200	1.60	0.36	1.62	200	0.63	0.08	0.66

Chapter 3

Efflux of Taurocholate (pmol/mg protein) from Cultured Rat Hepatocytes
(Figure 3-7 A & B)

A: Day-0, HBSS

<u>Time (min)</u>	<u>Mean</u>	<u>SD</u>
0.5	20.28	5.21
2	87.12	9.85
5	105.84	12.59
10	129.69	6.83

A: Day-0, Calcium-Free HBSS

<u>Mean</u>	<u>SD</u>
23.24	4.59
71.22	4.04
92.92	5.57
150.53	19.20

B: Day-5, HBSS

<u>Time (min)</u>	<u>Mean</u>	<u>SD</u>
0.5	22.58	2.49
2	31.29	1.88
5	48.38	3.04
10	57.05	4.76

B: Day-5, Calcium-Free HBSS

<u>Mean</u>	<u>SD</u>
28.70	5.98
60.88	0.77
90.93	3.50
93.59	6.17

Chapter 3

Efflux of Salicylate (pmol/mg protein) from Cultured Rat Hepatocytes
(Figure 3-7 C & D)

C: Day-0, HBSS

<u>Time (min)</u>	<u>Mean</u>	<u>SD</u>
0.5	28.59	1.35
2	43.71	2.23
5	56.19	0.45
10	59.82	3.96

C: Day-0, Calcium-Free HBSS

<u>Mean</u>	<u>SD</u>
19.18	6.77
52.75	5.77
54.97	3.51
65.85	6.20

D: Day-5, HBSS

<u>Time (min)</u>	<u>Mean</u>	<u>SD</u>
0.5	19.42	8.00
2	53.92	9.22
5	70.30	9.59
10	77.67	7.25

D: Day-5, Calcium-Free HBSS

<u>Mean</u>	<u>SD</u>
31.70	6.32
60.12	6.84
75.68	7.41
81.29	4.93

Chapter 3

Cumulative Uptake of Taurocholate (pmol/mg protein) in Cultured Rat Hepatocytes (Figure 3-8 A & B; Figure 5-5)

A: Day-0, HBSS

Time (min)	Mean	SD
0.5	66.06	12.14
2	192.75	18.35
5	255.87	25.39
10	338.09	19.57

A: Day-0, Calcium-Free HBSS

Mean	SD
43.09	4.11
144.49	14.69
223.49	17.52
304.22	27.20

B: Day-5, HBSS

Time (min)	Mean	SD
0.5	26.51	3.93
2	59.48	6.77
5	102.72	9.83
10	121.60	6.69

B: Day-5, Calcium-Free HBSS

Mean	SD
14.26	2.95
31.80	5.73
44.67	3.87
51.36	7.35

Chapter 3

Cumulative Uptake of Salicylate (pmol/mg protein) in Cultured Rat Hepatocytes
(Figure 3-8 C & D; Figure 5-2)

C: Day-0, HBSS

<u>Time (min)</u>	<u>Mean</u>	<u>SD</u>
0.5	9.30	0.93
2	12.26	2.82
5	15.21	3.39
10	20.77	5.96

C: Day-0, Calcium-Free HBSS

<u>Mean</u>	<u>SD</u>
10.95	0.88
14.64	1.56
21.06	5.57
25.22	9.18

D: Day-5, HBSS

<u>Time (min)</u>	<u>Mean</u>	<u>SD</u>
0.5	8.13	0.80
2	15.37	2.78
5	25.68	3.49
10	26.00	2.60

D: Day-5, Calcium-Free HBSS

<u>Mean</u>	<u>SD</u>
11.15	1.58
17.36	4.64
25.31	3.48
27.87	4.38

Chapter 3

Cumulative Uptake of Taurocholate (pmol/mg protein) in Sandwich-Cultured Rat Hepatocytes (Figure 3-9 A)

Uptake in HBSS

<u>Time (hr)</u>	<u>Mean</u>	<u>SD</u>
1	366.1	2.9
2	356.5	2.2
5	350.6	5.3
24	267.2	2.9
48	220.0	3.1
72	131.2	1.9
96	87.6	3.0
120	86.2	3.9

Uptake in Calcium-Free HBSS

<u>Time (hr)</u>	<u>Mean</u>	<u>SD</u>
1	340.1	11.6
2	327.3	3.0
5	321.4	7.9
24	209.7	3.7
48	127.6	2.1
72	60.9	2.1
96	29.3	5.5
120	36.0	0.6

Chapter 3

Biliary Excretion Index (%) of Taurocholate in Sandwich-Cultured Rat Hepatocytes
(Figure 3-9 B)

<u>Time (hr)</u>	<u>Mean</u>	<u>SD</u>
1	7.5	3.7
2	7.5	1.1
5	8.9	1.0
24	21.7	1.9
48	41.3	1.4
72	54.0	1.0
96	63.8	6.9
120	58.8	1.9

Chapter 4

Effects of Calcium on Taurocholate (25 μ M) Uptake (pmol/mg protein) in Freshly Isolated Rat Hepatocytes (Figure 4-1)

Time (min)	HBSS, 37°C		Calcium-Free HBSS, 37°C	
	Mean	SD	Mean	SD
0.25	1.61	0.20	1.58	0.17
0.5	3.24	0.17	3.06	0.34
1	4.70	0.50	3.96	0.53
2	6.41	0.80	5.78	0.11
5	7.33	0.84	7.05	0.72
10	8.83	0.92	8.14	0.12

Chapter 4

Effects of Calcium Depletion on Taurocholate Uptake (pmol/mg protein) in Sandwich-Cultured Rat Hepatocytes (Figure 4-4 A)

<u>Time (min)</u>	<u>Mean</u>	<u>SD</u>
0	104.79	2.87
1	66.10	5.33
2	60.05	7.95
5	60.68	3.02
10	46.87	11.01
20	44.88	7.11
60	28.89	6.03

Effects of Incubation of Sandwich-Cultured Rat Hepatocytes in HBSS on Taurocholate Uptake (pmol/mg protein, Figure 4-4 B)

<u>Time (min)</u>	<u>Mean</u>	<u>SD</u>
Control	107.82	4.89
0	47.23	11.37
5	58.58	11.31
10	68.03	4.38
20	82.10	10.56
60	84.95	12.21

Chapter 4

Cumulative Uptake of Taurocholate in Sandwich-Cultured Rat Hepatocytes (Figure 4-6)

Con (μ M)	Time (min)	Uptake in HBSS		Predicted	Uptake in Calcium-Free HBSS		Predicted
		Observed			Observed		
		Mean	SD		Mean	SD	
1	0.25	12.3	0.4	10.2	7.9	1.4	8.3
1	0.5	18.8	2.2	19.7	9.5	0.1	13.7
1	1	28.4	1.9	35.6	18.0	0.1	19.5
1	2	45.4	3.2	55.5	21.3	0.2	22.7
1	5	80.8	0.8	70.9	33.0	0.4	23.8
1	10	96.7	3.8	72.3	43.5	1.9	23.8
10	0.25	88.0	8.7	77.6	57.3	3.7	64.1
10	0.5	160.6	9.2	150.4	87.9	11.8	107.2
10	1	267.6	22.5	274.0	153.2	13.5	158.4
10	2	365.1	12.3	435.0	187.2	6.5	198.5
10	5	572.0	17.9	571.0	272.4	11.3	214.3
10	10	617.1	17.1	584.4	292.4	24.1	214.7
20	0.25	151.7	15.4	122.9	82.5	11.8	101.9
20	0.5	269.8	22.9	238.3	143.9	10.3	172.3
20	1	374.7	26.1	436.1	228.8	13.3	260.6
20	2	630.8	15.1	700.7	318.8	12.8	340.1
20	5	886.9	114.1	943.4	402.6	93.6	384.3
20	10	1050.4	136.2	972.2	506.2	52.5	387.0
50	0.25	272.5	10.9	189.1	144.6	11.0	157.9
50	0.5	414.7	38.4	367.3	243.7	8.3	270.3
50	1	733.2	28.4	675.9	392.3	40.9	421.7
50	2	1005.2	19.1	1104.8	432.3	10.1	582.9
50	5	1717.6	125.3	1559.0	682.4	71.2	721.3
50	10	1704.0	203.4	1643.4	832.5	105.9	745.5
100	0.25	365.0	39.3	230.5	202.5	28.7	193.1
100	0.5	453.8	66.4	448.1	300.7	37.5	333.2
100	1	984.0	91.2	827.2	439.5	103.4	528.9
100	2	1715.2	62.5	1366.2	726.9	81.7	755.7
100	5	1907.7	179.2	1994.6	939.8	288.4	999.6
100	10	1964.0	179.2	2160.3	1209.3	186.7	1072.1

Chapter 5

Cumulative Uptake of Inulin (pmol/mg protein) in Cultured Rat Hepatocytes
(Figure 5-1 A & B)

A: Day-0, HBSS			A: Day-0, Calcium-Free HBSS	
<u>Time (min)</u>	<u>Mean</u>	<u>SD</u>	<u>Mean</u>	<u>SD</u>
0.5	-0.26	0.36	0.40	0.73
2	-0.14	0.58	0.79	0.75
5	1.24	1.26	1.69	0.26
10	-0.72	0.63	1.93	2.37

B: Day-4, HBSS			B: Day-4, Calcium-Free HBSS	
<u>Time (min)</u>	<u>Mean</u>	<u>SD</u>	<u>Mean</u>	<u>SD</u>
0.5	-0.76	0.30	0.20	0.33
2	-1.14	0.98	-0.82	0.34
5	0.34	0.34	1.52	2.54
10	0.81	1.55	0.20	2.24

Chapter 5

Cumulative Uptake of Methotrexate (pmol/mg protein) in Cultured Rat Hepatocytes
(Figure 5-3 A & B)

A: Day-0, HBSS			A: Day-0, Calcium-Free HBSS	
Time (min)	Mean	SD	Mean	SD
0.5	1.71	0.49	1.51	0.81
2	4.61	0.57	4.80	1.41
5	11.37	1.49	8.89	0.97
10	16.82	0.60	15.16	1.13

B: Day-4, HBSS			B: Day-4, Calcium-Free HBSS	
Time (min)	Mean	SD	Mean	SD
0.5	0.69	0.33	0.76	0.68
2	3.58	1.69	1.33	0.91
5	5.53	0.84	3.04	1.96
10	9.42	1.07	4.32	2.26

Chapter 5

Cumulative Uptake of [D-Pen2,5]enkephalin (pmol/mg protein) in Cultured Rat Hepatocytes (Figure 5-4 A & B)

A: Day-0, HBSS

Time (min)	Mean	SD
0.5	389.83	21.95
2	1126.19	171.61
5	1845.17	150.46
10	2771.04	179.08

A: Day-0, Calcium-Free HBSS

Mean	SD
369.56	38.12
1168.68	151.80
1765.49	106.16
2622.52	206.84

B: Day-4, HBSS

Time (min)	Mean	SD
0.5	60.51	9.88
2	224.08	19.09
5	410.24	51.80
10	557.99	21.72

B: Day-4, Calcium-Free HBSS

Mean	SD
38.26	6.94
146.53	17.10
244.81	51.24
321.20	34.73

Chapter 5

Percentage of Dose Excreted in Bile and Biliary Excretion Index (Figure 5-6 A)

Substrate	%Dose in Bile		Biliary Excretion Index (%)	
	Mean	SD	Mean	SD
Inulin	0.0	0.0	0.0	0.0
Salicylate	0.0	0.0	1.0	0.0
Methotrexate	60.0	2.5	55.4	18.3
Enkephaplin	70.0	4.0	42.4	6.5
Taurocholate	99.0	0.0	56.4	5.2

In Vivo and In Vitro Biliary Clearance (ml/min per kg body weight; Figure 5-6 B)

Substrate	Intrinsic In Vivo Biliar Clearance		In Vitro Biliary Clearance	
	Mean	SD	Mean	SD
Inulin	0.0	0.0	0.0	0.0
Salicylate	0.0	0.0	1.0	0.0
Methotrexate	17.3	0.8	4.1	1.0
Enkephaplin	34.4	3.2	12.6	2.2
Taurocholate	116.9	N/A	56.2	6.0

Chapter 5

Cumulative Uptake of 264W94 (pmol/mg protein) in Cultured Rat Hepatocytes
(Figure 5-8 A & B)

A: Day-0, HBSS

<u>Time (min)</u>	<u>Mean</u>	<u>SD</u>
0.5	360.8	36.4
2	724.7	30.6
5	801.1	72.7
10	861.5	35.6

A: Day-0, Calcium-Free HBSS

<u>Mean</u>	<u>SD</u>
352.6	40.4
722.0	44.6
749.5	52.1
833.3	65.4

B: Day-4, HBSS

<u>Time (min)</u>	<u>Mean</u>	<u>SD</u>
0.5	464.8	125.4
2	858.6	42.6
5	922.5	104.7
10	1104.4	81.3

B: Day-4, Calcium-Free HBSS

<u>Mean</u>	<u>SD</u>
300.7	28.3
870.0	45.5
868.3	100.8
1034.3	46.4

Chapter 5

Cumulative Uptake of 2169W94 (pmol/mg protein) in Cultured Rat Hepatocytes
(Figure 5-9 A & B)

A: Day-0, HBSS

<u>Time (min)</u>	<u>Mean</u>	<u>SD</u>
0.5	295.8	72.2
2	633.8	75.4
5	824.0	105.1
10	999.5	105.4

A: Day-0, Calcium-Free HBSS

<u>Mean</u>	<u>SD</u>
275.3	85.5
612.9	79.8
781.0	96.9
953.3	141.2

B: Day-4, HBSS

<u>Time (min)</u>	<u>Mean</u>	<u>SD</u>
0.5	252.4	12.9
2	440.4	73.2
5	449.0	38.6
10	441.8	18.2

B: Day-4, Calcium-Free HBSS

<u>Mean</u>	<u>SD</u>
257.7	14.9
366.6	102.5
288.7	73.1
261.1	43.2